IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Aberg *et al.*

Confirmation No.: 3537

Serial No.: 09/447,218

Group Art Unit: 1623

Filed: November 23, 1999

Examiner: L. Crane

For: METHODS FOR TREATING URTICARIA USING DESCARBOETHOXYLORATADINE

Attorney Docket No.: 4821-362-999 (JD 208423-999361)

DECLARATION OF DR. PAUL M. TARANTINO, JR.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, PAUL M. TARANTINO, JR., Ph.D., declare and state as follows:

1. I am a citizen of United States and reside at 61 Summer Lane, Holden, MA 01520.

2. I received my Bachelor of Science degree in Biology from Villanova University, Villanova, PA in 1993. I received my Ph.D. degree in Pharmacology from University of Massachusetts Medical School, Worcester, MA in 1998.

3. After receiving my Ph.D., I was employed as a Staff Scientist, and then a Senior Scientist at GLSynthesis Inc. in Worcester, MA, from 1998 to 2001. In 2001, I was promoted to Director of Pharmacology at GLSynthesis Inc. and held that position until early 2002. In February 2002, I joined Sepracor Inc. ("Sepracor"), Marlborough, MA, as an Associate Director of Safety Pharmacology. Currently, I am Director of Safety Pharmacology at Sepracor. I have published in various peer-reviewed journals and presented my research at national and international meetings. A copy of my curriculum vitae is attached hereto as **Exhibit 1**.

4. Currently as the Director of Safety Pharmacology at Sepracor Inc., I am intimately involved in the process of drug development. My duties include the design and implementation of non-clinical safety programs including: study design, dosage selection, study initiation and monitoring. Non-clinical safety programs include, but are not limited to, *in vitro* assays and *in vivo* (*e.g.*, animal) studies. I regularly review reports concerning non-

clinical safety, and am involved in risk assessment and regulatory decisions concerning Sepracor's drug candidates. In particular, I extensively deal with toxicology and safety pharmacology related matters, including reviewing, maintaining and tracking records for all non-clinical toxicology and safety pharmacology studies and results at Sepracor.

5. I have reviewed the specification and claims of the present application. I have focused particular attention on Example 4 of the specification. In addition, I have read the Office Action dated August 8, 2005, attached hereto as **Exhibit 2**, and specifically, reviewed the Examiner's comments concerning Example 4. (*See* pages 8-9 of **Exhibit 2**).

6. I understand that the Examiner questions the significance of the data reported in Example 4 of the present application, in comparison with the data in Brandes *et al.*, *Journal of the National Cancer Institute*, 86(10): 770-775 (1994), in the context of what was known about certain potential adverse effects associated with loratadine and its metabolite descarboethoxyloratadine ("DCL") prior to December 30, 1994, the priority date of the present application. In particular, the Examiner states that it is unclear whether Example 4 of the present application shows "a real or imaginary difference in the capacity to promote neoplastic tissue growth in actual neoplasms" because "Brandes provides an examples [*sic*] of tests against real neoplastic-disease-infected cells while the data in the instant disclosure does not appear to provide a parallel disclosure." (**Exhibit 2**, page 9).

7. To assess the issues raised by the Examiner, I have reviewed the following additional materials:

1) Gleichmann *et al.*, "Immunotoxicology: supressive and stimulatory effects of drugs and environmental chemicals on the immune system," *Arch. Toxicol.*, 63(4): 257-273 (1989) ("Gleichmann"), attached hereto as **Exhibit 3**;
2) Vos, "Screening and Function Studies in Immunotoxicity Testing," *Vet. Q.*, 3: 190-195 (1981) ("Vos"), attached hereto as **Exhibit 4**;
3) Brandes *et al.*, "Enhanced Cancer Growth in Mice Administered Daily Human-Equivalent Doses of Some H_1-Antihistamines: Predictive In Vitro Correlates," *Journal of the National Cancer Institute*, 86(10): 770-775 (1994) ("Brandes"), attached hereto as **Exhibit 5**; and

4) Declaration of William W. Storms, M.D. ("Storms Declaration"), attached hereto as **Exhibit 6**.

8. I am, and have been prior to the preparation of this declaration, familiar with immunotoxicity assays such as those described in Vos and Gleichmann, which are discussed in more detail below.

I. Example 4 Shows that Descarboethoxyloratadine is Less Immunotoxic Than Loratadine

9. Immunotoxicity, in a pharmacological sense, is the degree to which a drug impairs a patient's immunological functions by, for example, suppressing the patient's immune system. (*See, e.g.*, Gleichmann, **Exhibit 3**, page 258, left column). Such toxicity or immunosuppressive effect of a drug candidate or a drug is seriously undesirable or unwanted because it impairs a patient's natural immune system and may render the patient susceptible to various infections and diseases, or expose the patient to an elevated risk of developing malignancy. (*Id.*, Abstract). Therefore, it is, and was prior to December 30, 1994, common to assess a compound's immunotoxicity when developing a drug.

10. One of the most well-accepted assays for assessing a compound's immunosuppressive effect is lymphocyte mitogenesis assay, similar to what is disclosed in Example 4 of the present specification. (*See, e.g.*, Vos, published in 1981, **Exhibit 4**, page 193). The assay disclosed in Example 4 in the present specification is designed to assess the degree of mitogenesis in cells that are treated with a specific mitogen for T cells (*e.g.*, concanavalin A) in the presence of a test compound, which, in the case of the instant application, is loratadine or DCL. The degree of inhibition of mitogenesis by the test compound correlates to immunotoxicity of that compound. In other words, the more inhibitory a compound is of mitogenesis, the more immunotoxic it is, and thus, that compound is less desirable as a drug candidate.

11. As shown in Example 4 of the present specification, IC_{50} values for inhibition of concanavalin A induced stimulation of lymphocytes were determined to be 1.0 μM and 5.6 μM for loratadine and DCL, respectively. Therefore, the results show that DCL is about 5-6 fold <u>less</u> potent in inhibiting mitogenesis, and thus less immunotoxic, than loratadine. Consequently, I would have concluded, based on the disclosure of Example 4 of

the present specification, that DCL would be more desirable as a drug candidate than loratadine with respect to immunotoxicity.

II. Example 4 Shows that Descarboethoxyloratadine is Less Likely to Promote Tumor than Loratadine

12. In addition to the immunotoxicities of DCL and loratadine, it would have been reasonable to conclude that Example 4 demonstrates that DCL is less likely to promote tumor growth than loratadine. This conclusion is based in part upon my review of Brandes, as explained below.

13. In Brandes, an *in vivo* assay for tumor promotion was performed, and the results were correlated with those obtained from five *in vitro* assays in an effort to devise a simple method that could provide useful information during preclinical development as to whether a compound would promote tumor growth. (Brandes, **Exhibit 5**, page 771, left column). In essence, Brandes was attempting to develop an *in vitro* surrogate model for evaluating the tumor promoting potential of a test article. One of the five *in vitro* assays performed in Brandes was lymphocyte mitogenesis test, which is virtually identical to what is disclosed in Example 4 of the present specification. (*Id.*, page 771, right column).

14. As a first step, the potencies of five antihistamines (*i.e.*, loratadine, astemizole, cetirizine, hydroxyzine, and doxylamine) in affecting tumor growth in rodents were examined in Brandes. (*Id.*, page 771, left column). The order of potencies in promoting tumor growth was determined to be: loratadine $\approx$ astemizole $\geq$ hydroxizine $\geq$ doxylamine $\approx$ cetirizine. (*Id.*, page 774, legend to Table 2).

15. Next, the potencies of the same five antihistamines in five different *in vitro* assays, one of which is the lymphocyte mitogenesis assay disclosed in Example 4, were examined. (*Id.*, page 771, left column). The results obtained from each of these five *in vitro* assays were correlated with those obtained from the *in vivo* assay described above. Upon correlating the results, the authors of Brandes concluded that "a significant correlation was observed between the rank order of potency of the antihistamines in <u>all five in vitro assays</u> and their rank order to enhance tumor growth in vivo." (*Id.*, page 772, right column) (emphasis added).

16. In particular, Brandes showed that the order of potencies of the five antihistamines, when determined by the lymphocyte mitogenesis test, was: loratadine $\geq$

astemizole ≥ hydroxyzine ≥ doxylamine ≥ cetirizine. (*Id.*, page 774, Table 2). Thus, the order of potencies of the five antihistamines determined using lymphocyte mitogenesis assay indeed correlated well to the order of potencies for tumor promotion determined using *in vivo* assay. Therefore, Brandes showed that an antihistaminic compound which is more immunotoxic, as determined by the lymphocyte mitogenesis test, is likely be more potent in promoting tumor.

17. Without being limited to a particular mechanism of action, given the results for antihistamines including loratadine in Brandes, it is my opinion that the immunotoxocity of loratadine or DCL was thought to correlate well to its tumor promotion potential prior to December 30, 1994. This is based in part on the fact that a compound with a higher immunotoxicity could interfere with the patient's immune defense against tumor, thereby promoting the growth of tumor. Conversely, a compound with a lower immunotoxicity could enable the patient's immune system to provide resistance against the growth of tumor.

18. As discussed above, Example 4 of the present specification indicates that DCL is 5 to 6 fold less immunotoxic than loratadine, when immunotoxicity is determined by the lymphocyte mitogenesis assay. Furthermore, it would have been reasonable to conclude that Example 4 demonstrates that DCL is <u>less potent in promoting tumors</u> than loratadine for the reasons discussed above. Consequently, I would have believed, based on the disclosure of Example 4 of the present specification, that DCL is a more desirable drug candidate than loratadine.

III. Brandes Suggests that Certain Non-sedating Antihistamines are More Immunotoxic and Potent in Promoting Tumor Growth than Others

19. The test results disclosed in Brandes, which was published prior to December 30, 1994, taught that loratadine and astemizole were most immunotoxic and most potent in promoting tumor among the five antihistamines tested. In addition, it was well-known that loratadine and astemizole were considered as members of a class of compounds known as "piperidine H_1 antagonists," as stated in Storms Declaration (*See* **Exhibit 6**, paragraph 13). Consequently, Brandes would have suggested to me that "piperidine H_1 antagonists," as a class, of which DCL is a member, are likely be more immunotoxic and potent in promoting tumor, as compared with other classes of antihistamines.

20. In sum, it is my opinion that: 1) the disclosure in Example 4 of the present application showed that DCL is less immunotoxic than loratadine, thereby demonstrating that DCL is more desirable as a drug candidate; and 2) the disclosure in Example 4, together with the teaching of Brandes, would have suggested that DCL is less potent in promoting tumor than loratadine, thereby demonstrating that DCL is more desirable as a drug candidate.

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make those statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: November 28, 2005

PAUL M. TARANTINO, JR., Ph.D.

Paul M. Tarantino Jr., Ph.D.
61 Summer Lane
Holden, MA 01520
Home Phone: (508) 829-4610
Work Phone: (508) 357-7527
Email: ptaran1@charter.net

Professional Experience

Sepracor Inc. Jan. 2005 to Present
Marlborough, MA 01752
Director, Safety Pharmacology

Sepracor Inc. Feb. 2002 to Dec. 2004
Marlborough, MA 01752
Associate Director, Safety Pharmacology

- Actively participate on drug development teams, including the design and implementation of the nonclinical safety programs, study design, dosage selection, study initiation and monitoring, report review and finalization, risk assessments, and regulatory interactions.
- Provide the nonclinical safety pharmacology support to multiple development projects and pre-development projects.
- Determine the design and timing of studies, involving the best available relevant consultants and validating new technologies when appropriate.
- Initiate, monitor and report all required studies in time to support project and submission goals.
- Write report summaries and project updates, as needed by the teams and/or management, and prepare relevant parts of regulatory submissions and supporting/defending them at the FDA as needed.
- Support organizational needs of the department and/or the teams according to special skills, opportunities and assignments.
- Be the 'on-call' safety pharmacologist for all projects, consulting to supervisors, physicians, regulatory staff, and program directors.
- Maintain tracking records of all nonclinical toxicology and safety pharmacology studies and results.
- Support Toxicology Department as needed during periods of understaffing (currently toxicologist on one major development program and two research programs).
- Maintain working knowledge of all new and developing regulatory guidances pertaining to nonclinical safety evaluations.
- Involved in the preparation of; annual reports for all development programs, investigator brochures, responses to FDA queries, INDs and three NDAs. Attended Type A, Type C and pre-NDA meetings with FDA.

GLSynthesis Inc. Feb. 2001 to Feb. 2002
Worcester, MA 01605
Director of Pharmacology

- Directed the research and daily activities of four Ph.D. level scientists in a non-GLP pre-clinical drug research, both for contract and internal research and development. Under my direction these scientists performed routine pharmacokinetic and acute toxicological testing of novel compounds, tested the effects of compounds *in vitro* in a variety of isolated tissues and established models for testing (among others): the effect of compounds on QTc in anesthetized guinea pigs, the effect of compounds on various smooth muscle

responses *in vivo* (including effects on mean arterial pressure, and urinary bladder spasticity), the effect of compounds on erectile function in rats and the effect of compounds on intestinal motility.
- Ultimately responsible for the design and conduct of experiments, correspondence with clients and the writing of reports and grant applications.
- Reported directly to the company President.
- Analyzed and interpreted data accumulated by three staff scientists and one senior scientist using appropriate statistical methods and software and expertise.
- Participated on project teams, presented accumulated data and selected compounds for further study.
- Responsible for report writing for both contract research and internal discovery/research and development projects.
- Presented data at internal and external scientific review committee meetings.
- Developed four *in vivo* models for evaluating the effects of novel compounds on Gram-positive bacterial infection.

GLSynthesis Inc. March 1998 to Jan. 2001
Worcester, MA 01605
Staff Scientist then Senior Scientist

- Directed the research of one to two Ph.D. level scientists.
- Responsible for the design and conduct of experiments, correspondence with clients and writing of reports.
- Participated in writing grant applications.
- Prepared budgets for both grant and contract work.
- Trained new staff in the use of laboratory equipment and techniques.
- Analyzed data accumulated by the pharmacology staff using appropriate statistical methods and software and expertise.
- Participated on project teams, presented data and assisted in the selection of compounds for further study.
- Responsible for report writing for both contract research and the internal discovery/research and development projects.
- Developed guinea pig model for evaluating topical anesthetics.
- Tested the effect of a variety of novel compounds on isolated tissues *in vitro*.
- Performed routine pharmacokinetic and toxicological testing of new compounds (IV, SC, PO, IP, IM).
- Established HPLC conditions and performed HPLC analysis of blood and urine for the presence of parent compound and/or metabolites.
- Developed a model for testing the effect of compounds on mucociliary transport in bovine trachea *in vitro*.
- Developed a model for the testing of antihistamines *in vivo* (croton oil induced ear inflammation).
- Tested the effect of various compounds on human sperm motility *in vitro*.

University of Massachusetts Medical School June 1993 to Feb. 1998
Worcester, MA 01655
Graduate Research Assistant

- Trained new technicians and students in the use of laboratory equipment and techniques.
- Analyzed data and designed experiments.
- Participated in the writing of scientific papers and grants.
- Presented work at department seminars and scientific meetings.
- Synthesized a series of N3-substituted 6-anilinouracils.
- Overexpressed and purified DNA polymerase III from two Gram-positive organisms.
- Performed *in vitro* biochemical assays measuring the effect of novel compounds on DNA replication by purified enzymes, in crude extracts and in permeabilized cells.
- Performed *in vitro* assays to measure the antibacterial effect of novel compounds (MICs).

- Performed routine pharmacokinetic and toxicological testing of new compounds (IV, PO, IP).
- Established HPLC conditions and performed HPLC analysis of blood and urine for the presence of parent compound and/or metabolites.
- Performed SDS-PAGE and Western blotting.
- Tested novel compounds in a pneumococcal lung infection model.

Additional Responsibilities/Professional Experience:

- Principal Investigator on a Phase I SBIR grant from the National Institutes of Health (NIH) titled "Hybrid Molecules Designed to Enhance Antibiotic Activity" (Grant No. 1R43GM060828-01). This project advanced and two Phase II applications were submitted and grants awarded, both entitled "Hybrid Molecules Designed to Enhance Antibiotic Activity (Grant No. 2R44GM060828-02, awarded to P. Tarantino and subsequently assigned to G.E. Wright and Grant No. 5R44GM060828-03 awarded to G.E. Wright)
- Involved in the writing of, and served as the Lead Pharmacologist on additional SBIR grants from NIH for internal research and development projects:
    - "Gram+ Antimicrobials Targeted to DNA Polymerase III", Grant No. 2R44AI041260-02A1, Awarded to G.E. Wright
    - "Drugs to Prevent Recurrent Herpesvirus Infections", Grant No. 2R44AI043170-02, Awarded to G.E. Wright
    - "Novel Drugs to Treat Urinary Incontinence", Grant No. 2R44AG015259-02, Awarded to J. Chen
    - "New Dermal Anesthetics", Grant No. 1R43AR046396-01 and 2R44AR046396-02A1, Awarded to V. Ciofalo

- During time at both UMMS and GLSynthesis, responsible for interactions with the Institutional Animals Care and Use Committee (IACUC) at the University of Massachusetts Medical School. Principal Investigator on approved animal protocols using mice, rats, Guinea pigs and rabbits.
- Attended professional training courses on history and implementation of FDA GLP regulations.
- Attended meetings of the Cardiotoxicity Biomarker Expert Working Group at FDA
- On the roster of the PhRMA Biomarkers and Surrogate Endpoints Work Group

Professional Affiliations:

Safety Pharmacology Society

Education:

University of Massachusetts Medical School, Worcester, MA *1993 to 1998*

Ph.D. Pharmacology

Thesis: Development of the Antibiotic Potential of a Unique Family of DNA Polymerase Inhibitors

Villanova University, Villanova, PA *1989 to 1993*

B.S. Biology

Publications/Presentations:

M.H. Barnes, P.M. Tarantino Jr., P. Spacciapoli, N.C. Brown, H. Yu and K. Dybvig, DNA Polymerase III of Mycoplasma pulmonis: isolation and characterization of the enzyme and its structural gene, *pol C*, *Molec. Microbiol.* **13**, 843-854 (1994)

P.M. Tarantino, G.E. Wright and N.C. Brown, Novel Antibacterial Agents Targeted against the Gram+ DNA Replicase: Effect of N3 Modification on Anti-pol III and Antimicrobial Activity, Annual Meeting of the New England Pharmacologists, February 1996

P.M. Tarantino Jr., C. Zhi, J. Gambino, G.E. Wright and N.C. Brown, 6-Anilinouracil-based Inhibitors of Bacillus subtilis DNA Polymerase III: Antipolymerase and Antimicrobial Structure-Activity Relationships Based on Substitution at Uracil N3, *J. Med. Chem.* **42**: 11, 2035-2040 (1999)

P.M. Tarantino Jr., C. Zhi, G.E. Wright and N.C. Brown, Development of Novel Antimicrobials Targeted to DNA Polymerase III of Gram-positive Eubacteria, *Antimicrob. Agents Chemother.* **43**: 8, 1982-1987 (1999)

Y. Schwartz, P. Tarantino, T. Jerussi. (R)-Albuterol and (S)-albuterol exhibit differential effects of mucociliary transport velocity in calf trachea. Poster presentation, Chest Meeting, 2002, San Diego, CA

M.M. Butler, W.A. LaMarr, K.A. Foster, M.H. Barnes, D.J. Skow, P.T. Lyden, T.L. Bowlin, C. Zhi, Z. Long, A. Manikowski, W.C. Xu, P.M. Tarantino, K.A. Holm, G.E. Wright. Antibacterial Efficacy of Novel Anilinouracil/Fluoroquinolone Hybrids. Poster presentation, ICAAC Meeting 2003, Chicago, IL

P. Tarantino, N. Appleton, K. Lansdell. Effect of trazodone on hERG channel current and QT-interval. *Eur. J. Pharmacology* 510: 75-85 (2005)

C. Zhi, Z.Long, A. Manikowski, N.C. Brown, P.M. Tarantino, K. Holm, E.J. Dix, G.E. Wright, K.A. Foster, M.M. Butler, W.A. LaMarr, D.J. Skow, I. Motorina, S. Lamothe, and R. Storer. Synthesis and Antibacterial Activity of 3-Substituted-6-(3-ethyl-4-methylanilino)uracils. *J. Med. Chem.* In press.

Y. Schwartz, P.M. Tarantino Jr., T. Jerussi. (S)-Albuterol Negatively Impacts the Mucociliary Transport Effects of (R)-Albuterol *In Vitro.* In preparation

References:

Available upon request

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/447,218	11/23/1999	A.K. GUNNAR ABERG	4821-362	3537

7590 08/08/2005

PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

Washington DC

EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	PAPER NUMBER
1623	

DATE MAILED: 08/08/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Amendment Due 11/8/05 (JL)
Appeal Due 11/8/05 (JL)

Office Action Summary	Application No. 09/447,218	Applicant(s) ABERG ET AL.
	Examiner L. E. Crane	Art Unit 1623

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *May 18, 2005 (amdt)*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *34,36,38-40 and 50* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *34, 36, 38-40 and 50* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *5/18 & 6/15/2005*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

Claims **1-33, 35, 37 and 41-49** have been cancelled, no claims have been amended, the disclosure has not been amended, and new claim **50** has been added as per the response filed May 18, 2005. Two additional Information Disclosure Statements (2 IDSs) filed May 18, 2005 and June 15, 2005 have been received with all cited references. A declaration signed by William W. Storms, M.D. has also been received with Exhibits A, C and D complete, but with the documents listed on Exhibit B not all bibliographically complete. Documents listed in Exhibits B and D which are bibliographically complete have been cited on the attached PTO-892. But, references E-6, E-9, E-10, E-11 (No dates of publication, some author and publisher information incomplete) could not be made of record because these references did not have complete bibliographic information included for same on either the face of the document or in the listing of references provided by applicant or both. Resubmission is respectfully requested together with the missing bibliographic information preferably printed on a PTO-1449.

Claims **34, 36, 38-40 and 50** remain in the case.

The following is a quotation of 35 U.S.C. §103(**a**) which forms the basis for all obviousness rejections set forth in this Office action:

> "A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

Claims **34, 36, 38-40 and 50** are rejected under 35 U.S.C. §103(**a**) as being unpatentable over **Berkow et al.** (PTO-892 ref. **R**) in view of **Villani et al. '716** (PTO-1449 ref. **AE**).

The instant claims are directed to the treatment of urticaria (aka hives, a condition associated with allergic reactions) by the administration of an effective dosage of descarboethoxyloratadine (DCL) to a patient in need thereof. The dosage is further defined as 0.1 mg to less than about 10 mg per day or less than about 5 mg per day. Claim **36** further limits claim **34** by limiting the population of hosts to those "... humans with a higher than normal propensity for or incidence of cancer."

Berkow et al. discloses at p. 333, beginning in the third line under "**Treatment**," that " [s]ymptoms [of urticaria] usually can be relieved with an oral [dose of an] antihistamine ... ."

Villani et al. '716 discloses at column 1, lines 39-46 that descarboethoxyloratadine (DCL) and closely related compounds are effective antihistamines with the advantage of low CNS-related side effects, i.e. that DCL and relatives are non-sedative. Villani also discloses at column 8, lines 11-46 that the dosage range is about 1 mg to 40 mg for a 24 hour period and preferably from about 5 to about 10 mg over this time period (column 8, line 19) More generally the unit dosage is defined as "from 1 mg to 1000 mg according to the particular application" (column 8, lines 43-44).

The findings that
i) Villani et al.'s teaching that DCL and related compounds are known to be effective antihistamines,
ii) the teaching by applicant that DCL has the expected effect in the treatment of urticaria (hives) as predicted by Berkow et al.,
iii) the teaching of dosages ranges which overlap with the claimed dosage ranges, and
iv) the failure of applicant to establish statistically significant unexpected results (no error analysis of the data provided),
when taken together with the disclosure of Berkow et al. are deemed to establish that combination of the instant combination of references is properly motivated. These particular disclosures are also deemed to render the instant claimed subject matter lacking in any patentable distinction in view of the noted prior art.

Therefore, the instant claims directed to treatment of urticaria by the administration of the antihistamine DCL, including within the dosage ranges of the instant dependent claims, would have been obvious to one of ordinary skill in the art having the above cited references before him at the time the invention was made.

Applicant's arguments filed May 18, 2005 have been fully considered but they are not deemed to be persuasive.

Applicant argues that in Berkow is not applicable because only first generation antihistamines are listed as examples and furthermore that the prior art teaches that not all antihistaminic agents can be used to treat urticaria. After noting examiner's counter argument that even Berkow acknowledges that not all antihistamines are effective in every patient, applicant further argues that the instant claims are "not obvious because Berkow does not

disclose the use of DCL for the treatment of urticaria, and does not even suggest that all antihistaminic agents are effective in treating urticaria." (emphasis in original) Examiner respectfully disagrees with applicant's conclusion that "... Examiner is in complete agreement with Applicant that Berkow does not render obvious the treatment of urticaria with all known antihistamines." (emphasis in original) Applicant then concludes without more that Berkow is " ... nothing more than an invitation to experiment," without even considering the contents of the other primary reference. Applicant then leaps to the conclusion that because " ... an invitation to experiment is not a proper basis for an obviousness rejection, ... the rejection ... should be withdrawn." Examiner respectfully disagrees with this line of reasoning for the following reasons. First, the standard for obviousness does not require that a single reference must meet the standard for anticipation, i.e. that all elements of a rejection over prior art must be present in a single reference. Second, applicant alleges that the Berkow reference fails because it must teach the specific active ingredient specified by the instant claims. Again this is the standard for anticipation, not the obviousness standard.

Applicant then argues beginning in the first full paragraph of page 6 of the response that Villani does not remedy the deficiencies of Berkow. Applicant then cites at length portion of the Storm declaration to the effect that the side effects attributed by Dr. Storm to DCL were so serious that no one would have considered DCL to be a proper candidate for use as an antihistamine for the treatment of urticaria as of the earliest priority document filing date in 1994. Examiner respectfully disagrees for the following reasons. Looking first at the paragraphs 12-15 of the Storm declaration, examiner notes that applicant's arguments are based on Dr. Storm's explanation of the standards applied to Food and Drug Administration (FDA) proceedings concerning the process of obtaining US government permission to market a pharmaceutical. In particular examiner notes that in paragraph 14, Storm states that " ... I considered reports concerning the adverse effects of one [piperidine] H_1 antagonist to be relevant to the safety of other members of that class." While examiner commends Dr. Storm for his extensive experience in FDA proceeding as documented in his lengthy resume, examiner does not agree that what appears to be a "safe and effective" analysis applies in this forum (before the USPTO); patentability, except where there is clear evidence of inoperability, is an entirely different determination. Applicant's have cited paragraph 17 of the instant declaration, a paragraph which appears to represent the professional opinion of Dr. Storm, but is presented without any data whatsoever to support his allegation that "cardio-toxicity,"

"personality changes" and "tumour growth" are associated with the administration of any level of dosage of DCL. Examiner has carefully reviewed the extensive list of references submitted by Dr. Storm and finds that no combination of these references can provide an adequate factual basis for concluding that DCL is inoperable as an antihistamine. Again examiner emphasizes that the standard for inoperability under 35 U.S.C. §101 is entirely different than those standards applied by the FDA in their proceedings.

Applicant concludes by arguing that the Berkow and Villani references are not properly applied under the statute and therefore that the rejection should be withdrawn. Examiner respectfully disagrees. The rejection has been maintained because the references cited teach generally that antihistamines are useful in the treatment of urticaria (Berkow) and that claims ***3 and 15*** of Villani '716 are directed specifically to the treatment of "allergic reactions," where the quoted term is, according to at least one medical dictionary, a synonym for the term "urticaria."

Examiner has reviewed in detail the declaration of Dr. William Storm. Dr. Storm is clearly knowledgeable in the medicinal antihistamine art, but the instant declaration appears to be beside the point. The instant declaration is of record in an application at the U. S. Patent and Trademark Office (USPTO), not the Food and Drug Administration (FDA). The USPTO is concerned with the disclosure and patenting of inventions, but the USPTO is not in the business of giving permission to market drugs (an FDA matter as suggested by the "safe and effective" language in declaration paragraph 22). Therefore, while Dr. Storm's concerns about side effects would probably be relevant to proceeding before the FDA as suggested by paragraphs 18 and 21 of the instant declaration, these concerns are not relevant to proceedings before the USPTO unless there is a factual disclosure (i.e. a substantial body of relevant test data) that the side effects are real (extrapolation of tests of other compounds, opinions and/or speculations are not enough), that said side effects are actually caused by the pharmaceutical in question, and that said side effects render the pharmaceutical in question completely unable to have the beneficial effect described in a patent claim (i.e. that the invention being claimed is inoperative under 35 U.S.C. §101). Because the instant declaration has not provided an adequate basis to support the conclusion that the prior art (Villani and Berkow) are (or were) inoperative, the instant declaration does not provide a disclosure which is sufficient to support a conclusion that the instant claims are patentable in view of said prior art; i.e. this declaration does not provide an adequate factual basis for disqualification of the cited prior art. Therefore,

examiner concludes that the instant declaration for the most part is not relevant to the question of patentability in the instant case.

Claims **34, 36, 38-40 and 50** are rejected under 35 U.S.C. §103(**a**) as being unpatentable over **Swinyard (II)** (PTO-892 ref. **TA**) and **Swinyard (I)** (PTO-892 ref. **SA**) in view of **Villani et al. '716** (PTO-1449 ref. **AE**) and further in view of **Brandes et al.** (PTO-892 ref. **RA**).

The instant claims are directed to the treatment of urticaria (aka hives) by the administration of an effective dosage of descarboethoxyloratadine (DCL) to a patient in need thereof and avoidance of the side effects thereof. The dosage is further defined as 0.1 mg to less than about 10 mg per day or less than about 5 mg per day.

Swinyard (II) discloses the utility of H_1-antihistamines in the treatment of urticaria at page 1124, column 1, line 4 of the third paragraph following the heading "Antihistamines." And at page 1130, column 2, this reference discloses the antihistamine azatadine maleate which is a very close structural relative of both loratadine and descarbethoxyloratadine (DCL), sharing with both the identical four ring molecular skeleton and similar antihistamine activity.

Swinyard (I) discloses at pages 778-782 (see page 779, paragraph beginning at column 1) that only certain H_2-antihistamines cause problems with liver P450 enzyme metabolism, but that selection of an alternative H_2-antihistamine is an effective way to avoid this difficulty.

Villani et al. '716 discloses at column 1, lines 39-46 that descarboethoxyloratadine (DCL) and closely related compounds are effective antihistamines with the advantage of low CNS-related side effects, i.e. that DCL and relatives are non-sedative. Villani also discloses at column 8, lines 11-46 that the dosage range is about 1 mg to 40 mg for a 24 hour period and preferably from about 5 to about 10 mg over this time period (column 8, line 19) More generally the unit dosage is defined as "from 1 mg to 1000 mg according to the particular application" (column 8, lines 43-44).

Brandes et al. discloses in the last sentence prior to the "CONCLUSION" that loratadine promotes the growth of two well known neoplasms. This issue is addressed by the instant disclosure at page 24, lines 20-25, with data which is at best incomplete because only one

comparison is made and because there is no statistical error analysis permitting determination of whether the difference suggested by the data is statistically significant.

The motivation to combine the above references is that each is directed to positive and/or negative effects observed following the administration of antihistamines. The primary reference, Swinyard (II) clearly is well motivated in combinations with the remaining references, because this reference discloses all of the side effects commonly associated with H_1-antihistamine administration, and how these may be avoided by substitution of alternative antihistamines, including an antihistamine which is a close structural relative of both loratadine and DCL.

The substitution of DCL for loratadine or its analogue azatadine is deemed to have been an obvious substitution of the ordinary practitioner seeking to optimize the treatment of urticaria as reported in Swinyard (II) in view of the disclosure of Villani et al. that DCL is, like loratadine, also an antihistamine. The additional guidance provided by the Swinyard (I) and Brandes references merely adds to the already extensive discussion in Swinyard (II) of the side effects observed following administration of medicinally appropriate dosages of numerous antihistamines, and therefore would have afforded the ordinary practitioner more than sufficient guidance to make an appropriate determination of whether or not to use DCL to treat urticaria in a given human host in need thereof. These particular disclosures are also deemed to render the instant claimed subject matter lacking in any patentable distinction in view of the noted combination of prior art.

Therefore, the instant claims directed to treatment of urticaria by the administration of the antihistamine DCL when appropriate in light of possible side effects, including within the dosage ranges of the instant dependent claims, would have been obvious to one of ordinary skill in the art having the above cited references before him at the time the invention was made.

Applicant's arguments filed May 18, 2005 have been fully considered but they are not deemed to be persuasive.

Beginning at the bottom of page 7 of the instant response applicant argues that Swinyard (II) is an inappropriate reference because " ... while 'enormous number [sic] of clinical conditions for which antihistamine drugs have been suggested,' these drugs 'vary from *effective* to *ineffective* in these conditions,' this [reference] clearly teaches that no

generalization can be made regarding a particular antihistamines efficacy in treating a particular disease, based on a different agent's efficacy." (emphasis in original) Examiner respectfully disagrees with the logic of this argument because again, applicant is asserting that a reference in an obviousness rejection requires meeting the standard of an anticipatory reference. Swinyard (II) is a reference cited in an obviousness rejection, not in an anticipation rejection. And applicant appears to have misstated the teachings of this reference. Examiner notes at page 1124, column 1, third paragraph following the heading **Antihistamines**, Swinyard states that " [t]he majority of these agents are *effective in perennial* and *seasonal allergic rhinitis, vasomotor rhinitis, allergic conjunctivitis, urticaria* and *angioedema, allergic reactions to blood and plasma, dermographism* and as adjuncts to conventional therapy in *anaphylactic reactions*." (italics in original) Therefore, applicant's conclusion that " ... Swinyard (II) would not have suggested that antihistamines in general ... can be used for the treatment of urticaria" is deemed to be a misreading and/or a misunderstanding of Swinyard (II).

In the second full paragraph at page 8 of the instant response, applicant argues that in view of page 590 of Goodman & Gilman (now cited as PTO-892 ref. **XC**) and paragraphs 12-17 of the Storm declaration that the suggestion of parallel pharmaceutical activity based on structural analogy was not properly a basis for alleging motivation to combine references. Applicant notes that allegations made by the Storm declaration as a proper basis for discussion of patentability. Examiner respectfully disagrees, referring applicant to the extensive analysis of the Storm declaration supra wherein examiner concluded that Dr. Storm was applying the "safe and effective" standard of the FDA, a standard not applicable to patentability determinations at the U.S. Patent and Trademark Office. Reviewing the contents of page 590, examiner notes that the last sentence of the second column states that "[t]hese [above noted] side effects are not observed with second generation H_1 antagonists, terfenadine, astemizole and loratadine," suggesting that the arguments of applicant and declarant Storm may be overly pessimistic as well as incomplete.

In the footnote at the bottom of page 8 of the instant response, applicant argues that Brandes is not properly part of the obviousness rejection on the basis that fear of said disclosure would not motivate the ordinary practitioner to use DCL in place of loratadine. Examiner respectfully disagrees, noting that the instant claim **36** includes a specific limitation to hosts less likely to suffer from neoplasms. Applicant's citing fear as a reason to avoid

looking for the facts is puzzling. The facts missing from the instant disclosure, error limits for the data concerning the difference in the ability of loratadine and DCL to promote certain neoplastic tissue growth, is not irrelevant, because said data would tell the ordinary practitioner whether the difference in neoplasm promotion between DCL and loratadine is statistically significant or not, an important consideration in determining how useful DCL would be as a replacement for loratadine, and a fact useful in determining whether claim **36** is properly supported based on the complete facts. Brandes provides an examples of tests against real neoplastic-disease-infected cells while the data in the instant disclosure does not appear to provide a parallel disclosure. Therefore, it is unclear to examiner whether claim **36** is reflective of a real or an imaginary difference in the capacity to promote neoplastic tissue growth in actual neoplasms as disclosed in test results of the Brandes et al. reference. However, examiner maintains that Brandes does suggest that there is a possible prior art basis for the limitation of claim **36** and therefore has been retained in the rejection.

In the first paragraph of page 9 response, citing the Storms declaration applicant argues that " ... those of ordinary skill in the art would not have been motivated to use DCL for the treatment of allergic disorders because prior known [structurally analogous] antihistamines ... were all known to cause serious adverse effects." Examiner respectfully disagrees because applicant and declarant Storms are again relying on a "safe and effective" type of analysis when the current debate is occurring in a non-FDA forum where this analysis is not directed to the appropriate standard. As noted above the standard for "inoperative" under 35 U.S.C. §101 is an entirely different analysis. Examiner remains convinced that the prior art presented by both applicant's representative and declarant Storms, individually or taken in sum, does not appear to require application of this type of analysis and the related rejection under §101.

In the second paragraph on page 9 of the instant response, applicant asserts that "Swinyard (I) and Villani add nothing to the substance of the rejection" initially because Swinyard (I) notes a side effect and that this and other side effects are commonly avoided by substitution of a different antihistamine, and that structurally similar compounds were reported to have similar side effects that the ordinary practitioner would not have been motivated to select DCL as a replacement of any other antihistamine. Secondly applicant argues that because Villani "merely discloses the crude antihistiminic activity of the compounds it discloses, [and] does not even provide that its compounds, much less DCL, can be used for the treatment of urticaria," and thereby implying that Villani is not a proper reference. On the first

argument examiner respectfully disagrees with applicant's conclusion because Swinyard (I) as described by applicant also teaches the freedom to substitute one antihistamine for another, side effects being only one of many reasons (reduced cost, improved selectivity, reduced dosage, etc.) why this might be done by the ordinary practitioner. On the second argument examiner also respectfully disagrees. Applicant is factually incorrect to imply the Villani does not disclose DCL; see Villani '716 at column 26, claim ***3*** wherein the complete chemical structure of DCL is provided And claim ***15*** of Villani '716 is directed to the treatment of "allergic reactions," a generic term which encompasses many conditions including urticaria; see Thomas et al., (PTO-892 reference **T**) wherein "urticaria" is defined at length as a term synonymous with a large and various array of "allergic reaction[s]." And finally applicant's referral to Villani's disclosure of "crude antihistaminic activity [of DCL]" is another reflection of applicant's repeated application of the wrong (FDA "safe and effective") standard as a basis for debating this and other issues. Villani '716's limited disclosure of antihistaminic activity is sufficient to establish that DCL and its fluoro analogue have utility as pharmaceutically active substances with the potential to be effective antihistamines in the treatment of allergic reactions.

Therefore, examiner has maintained this instant grounds of rejection.

Applicant's amendment's have necessitated the new grounds of rejection. Accordingly, **THIS ACTION IS MADE FINAL**. Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. §1.136(a).

A shortened statutory period for response to this final action is set to expire THREE MONTHS from the date of this action. In the event a first response is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 C.F.R. §1.136(a) will be calculated from the mailing date of the advisory action. In no event will the statutory period for response expire later than SIX MONTHS from the date of this final action.

Papers related to this application may be submitted to Group 1600 via facsimile transmission (FAX). The transmission of such papers must conform with the notice published

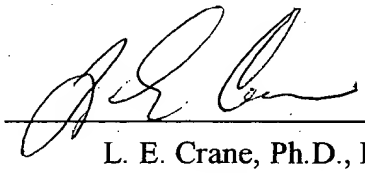
in the Official Gazette (1096 OG 30, November 15, 1989). The telephone number to FAX (unofficially) directly to Examiner's computer is 571-273-0651. The telephone number for sending an Official FAX to the PTO is 703-872-9306.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner L. E. Crane whose telephone number is **571-272-0651**. The examiner can normally be reached between 9:30 AM and 5:00 PM, Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. James O. Wilson, can be reached at **571-272-0661**.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is **571-272-1600**.

LECrane:lec
08/02/2005



L. E. Crane, Ph.D., Esq.
Primary Patent Examiner
Technology Center 1600

Arch Toxicol (1989) 63: 257–273

Archives of
Toxicology

Review article

Immunotoxicology: suppressive and stimulatory effects of drugs and environmental chemicals on the immune system

A discussion*

E. Gleichmann[1], I. Kimber[2], and I. F. H. Purchase[2]

[1] Division of Immunology, Medical Institute of Environmental Hygiene at the Heinrich Heine University of Düsseldorf, Auf'm Hennekamp 50, D-4000 Düsseldorf, Federal Republic of Germany
[2] Central Toxicology Laboratory, ICI plc, Alderley Park, Cheshire, SK10 4TJ, UK

Abstract. The fundamental characteristic of the adaptive immune system which has evolved in the vertebrates is the ability to recognise, and subsequently destroy, "foreign", and potentially harmful, antigens. The selective advantage which the immune system confers is the capacity to resist infectious, and possibly malignant, disease. It has been apparent for many years that individuals in whom immune function is impaired, due either to a congenital defect or to other factors such as treatment with certain immunosuppressive drugs, exhibit an increased susceptibility to infection and, in some cases, an elevated risk of developing at least some forms of malignancy. There is an increasing awareness from rodent studies that a variety of drugs and environmental chemicals have the potential to unintentionally impair components of the immune system. Risk assessment, based upon data from chemically induced changes in one or more parameters of immune function, is, however, dependent upon a knowledge of the functional reserve of the immune system. One of the objectives of the meeting from which this report derives was to examine what sources of information are available, and what experimental protocols can be employed, to permit accurate evaluation of immunological reserve. Although, under normal circumstances, the immune system selectively and specifically recognises foreign antigen, it is clear that the potential to recognise "self" is present and that in certain circumstances this potential is realised. Antibodies directed against normal tissue antigens have been shown to be associated with, and in some instances the presumptive cause of, "autoimmune" disease. There is a growing list of drugs and chemicals which are capable of eliciting autoantibodies and pathological autoimmune reactions. A second purpose of this meeting and of this report was to review the current state of knowledge regarding drug- and chemical-induced autoimmunity.

Key words: Autoimmunity – Environmental chemicals – Drugs – Immunosuppression – Immunostimulation

* This report results from a discussion sponsored and organised by the Advisory Subgroup in Toxicology (AST) of the European Science Foundations Standing Committee for the European Medical Research Councils and held at the ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Those taking part were: W. N. Aldridge (AST; Toxicology Unit, Medical Research Council Laboratories, Carshalton, UK); J. Descotes (Immunotoxicology Section, Department of Pharmacology, Alexis Carrel Medical Faculty, Lyon, France); P. Druet (Groupe de Recherches sur la Pathologie Rénale et Vasculaire, INSERM U28, Hôpital Broussais, Paris, France); S. Fossum (Anatomical Institute, University of Oslo, Oslo, Norway); E. Gleichmann (Division of Immunology, Medical Institute of Environmental Hygiene, Düsseldorf, FRG); M. Haeney (Department of Immunology, Clinical Sciences Building, Hope Hospital, Salford, UK); D. Henschler (AST; Department of Toxicology and Pharmacology, University of Würzburg, FRG); B. Holmstedt (AST; Department of Toxicology, Karolinska Institute, Stockholm, Sweden); R. W. G. Johnson (Department of Surgery, Manchester Royal Infirmary, Manchester, UK); I. Kimber (ICI Central Toxicology Laboratory; Alderley Park, Macclesfield, Cheshire, UK); R. Lauwerys (AST; Industrial and Medical Toxicology Unit, University of Louvain, Brussels, Belgium); F. Lembeck (AST; Department for Experimental and Clinical Pharmacology, University of Graz, Austria); N. Lery (Laboratoire de Medecine Legale et Toxicologie Medicale, Lyon, France); G. S. Panayi (Department of Medicine, Guy's Hospital Medical School, London, UK); A. H. Penninks (Department of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, Utrecht, The Netherlands); I. F. H. Purchase (AST; ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK); B. Ryffel (Sandoz A. G., Basle, Switzerland); B. Terracini (AST; Department of Pathology and Cancer Epidemiology, University of Turin, Turin, Italy); I. Todd (Department of Immunology, Middlesex Hospital Medical School, London, UK; now Department of Immunology, University Hospital, Queen's Medical Centre, Nottingham, UK); J. G. Vos (Laboratory for Pathology, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands); B. M. Vose (ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, UK); J. J. Weening (Department of Pathology and Internal Medicine, University of Leiden, Leiden, The Netherlands).

Offprint requests to: E. Gleichmann

Introduction

There is a growing awareness that a variety of chemicals have the potential to influence the functional activity of

the immune system. Induced changes in immunological status can be broadly divided into those in which immune function is impaired and those in which tissue-damaging allergic or autoimmune responses are initiated. The purpose of the meeting from which this report is derived was two-fold, firstly to examine what sources of information are available for determining the relationship between impairment of immune function and altered host resistance to infectious and/or malignant disease, and secondly to explore the current state of knowledge regarding the potential of drugs and chemicals to induce autoimmunity.

Chemically-induced impairment of immune function

During the last decade the field of immunotoxicology has attracted considerable attention and there now exists substantial literature, a review of which reveals that a variety of chemicals are able to impair the functional integrity of the immune system of rodents. Evaluation of the toxicological significance and human health implications of data obtained from experimental studies in rodents requires, however, a careful consideration of a number of factors. From a purely practical point of view it is relevant to consider whether there exist chemicals which, under certain conditions of exposure, are selectively immunotoxic, and would therefore escape detection in routine toxicological analyses. Although it is not unreasonable to suppose that selective immunotoxins exist, there are few examples in the literature. It is also pertinent to address the question of whether perturbations in immune function are reversible, and here again little information is available. Perhaps the major problem posed by experimental immunotoxicity studies is the relevance of quantitative or qualitative changes in one or more components of the immune system to the functional activity of the integrated mechanisms of host defence and the ability to resist infection and malignant disease. Of direct relevance to the relationship between empirical observations of dysfunction following chemical exposure and the integrity of host defence mechanisms is the extent to which the immune system possesses a functional reserve. There are two avenues of investigation which might be expected to yield information of value in assessing the functional reserve of the immune system. Firstly, in experimental systems, it is possible to examine directly the relationship between chemically induced perturbations of immune function and the ability to resist challenge with transplantable tumours and/or pathogenic micro-organisms. A second, and in our opinion, currently under-valued, source of information is available from the clinical and laboratory examination of congenital and acquired immune deficiency disorders in man.

The immune system as a target for toxicity

The ability of an organism to respond to foreign material is phylogenetically ancient and can be traced back to the protozoa and coelenterates. Such mechanisms of host resistance clearly offer important evolutionary advantages and in man there exist a number of "natural" (non-specific) defence mechanisms, such as the action of scavenger phagocytic cells, which play an important role in resistance to infectious disease. In addition to such natural protective mechanisms there has, in the vertebrates, evolved an exquisitely sensitive adaptive immune system in which there is a specific recognition of, and response to, foreign antigens. The cardinal features of the mammalian adaptive immune system are memory, specificity and the capacity to distinguish between self and non-self. It is the lymphocyte which plays a pivotal role in adaptive immune responses and which exhibits the properties of antigenic specificity, immunological memory and the ability to distinguish foreign, and potentially harmful, antigens from those normally expressed in the organism. Lymphocytes are clonally distributed with respect to antigenic specificity and each clone of lymphocytes possesses a unique membrane receptor for antigen. On primary exposure to foreign material those clones of lymphocytes which express complementary membrane receptors recognise and respond to antigenic determinants. The response comprises both division and differentiation. The induced prolifera-

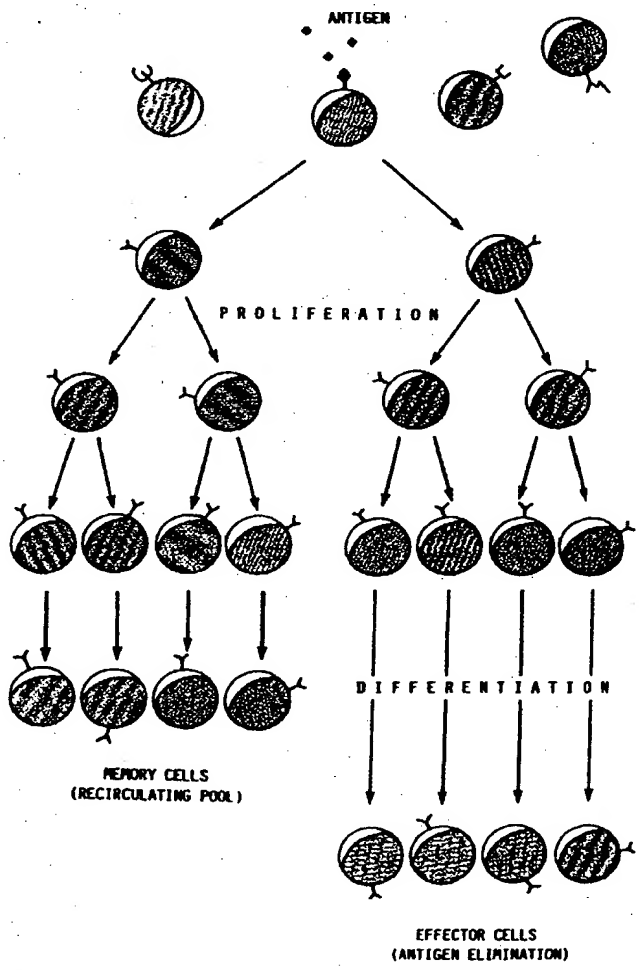


Fig. 1. Exposure to antigen results in the stimulation of those clones of lymphocytes (both T and B cells) which express complementary membrane receptors for antigen (clonal selection). Antigenic stimulation results in division (clonal expansion). A proportion of daughter lymphocytes differentiate into cells which either directly, or through the elaboration of effector molecules, eliminate antigen. The remainder of daughter lymphocytes fail to differentiate and constitute an expanded pool of cells (memory cells) which facilitate a more pronounced response following subsequent exposure to the same antigen

tion results in an expansion of those lymphocyte clones capable of recognising the stimulating antigen. Following division, a proportion of daughter lymphocytes undergo terminal differentiation into effector cells whose physiological role it is to eliminate antigen. The remaining daughter cells fail to differentiate and comprise the long-lived memory cells which provide an increased pool of antigen-sensitive lymphocytes that mount an accelerated and more aggressive immune response following secondary exposure to the same antigen (Fig. 1).

Superimposed upon the complexity of clonal distribution is the functional heterogeneity of lymphocyte populations. The two main classes of lymphocyte involved in adaptive immune responses are T and B lymphocytes. Following antigenic stimulation, B lymphocytes differentiate into plasma cells which possess the synthetic and secretory machinery to manufacture and export large amounts of antibody which has a specificity identical to that of the membrane antigen receptor expressed on the stimulated B lymphocyte. Antibodies are of particular importance in host resistance to extracellular bacterial infection. The effector cells into which antigen-stimulated T lymphocytes differentiate are those which are able to recognise and lyse infected host cells and those which elaborate a variety of soluble factors, known collectively as lymphokines. Lymphokines are responsible for a variety of important immune phenomena, including intercellular communication and the recruitment and activation of phagocytic cells. Effector T lymphocytes play a central role in resistance to infection by viruses and facultative intracellular bacteria where the effectiveness of antibody molecules is limited.

The last 20 years have witnessed significant and exciting advances in our understanding of the mechanism of action and control of immune responses, and it is now apparent that normal immunological function is dependent upon a series of sophisticated interactions at the molecular, cellular and tissue level. Thus, for instance, it became clear in the early 70s that functional sub-populations of T lymphocytes can be induced which promote (known as T helper cells) and regulate (T suppressor cells) immune responses. It is apparent that, in addition to providing mechanisms for the recognition and elimination of foreign antigens, the immune system incorporates a variety of elaborate internal checks and balances which together allow for efficient homeostasis.

A detailed account of the complex cellular and molecular events which together comprise normal immune function and control is clearly beyond the scope of this review. The important point is, however, that, in common with all finely-tuned biological systems, immune activity is potentially sensitive to disruption of normal function either through physiological or pathological change or following exposure to xenobiotics. One can envisage that relatively subtle changes in the delicate molecular and cellular fabric of immunity could create a functional imbalance causing a cascade of perturbations resulting in marked alterations in physiological function.

The importance of a functionally intact adaptive immune system is most clearly illustrated by the clinical consequences of severe congenital and acquired immune deficiency disorders in which one or more components of the immune system are affected. In the context of immunotoxicology it is relevant to consider whether the natural history of human immunodeficiency disorders provides information of value in assessing the functional reserve of the immune system.

Congenital and acquired immune deficiency disorders in man

The spectrum of immune deficiency disorders range from relatively minor changes such as the absence of an immunoglobulin class or sub-class to severe combined immunodeficiency which is characterised by the failure of T and B lymphocyte development and is immediately life-threatening (Table 1).

The primary manifestation of immunodeficiency is undue susceptibility to infections; infections that are more serious, more persistent, more unusual or recur more frequently than in the normal population. Infections in immunodeficiency states are characteristically related to the nature of the lesion. For instance, antibody deficiencies are typically associated with gram-positive bacterial infections, while patients with cellular immune deficiencies are

Table 1. Congenital and acquired immunodeficiency disorders[a]

A. Selected examples of congenital immunodeficiency disorders

Disorder	Immunological defect	Primary symptoms
Severe combined immunodeficiency disease (SCID) (Glanzmann and Rinicker 1950; Gitlin et al. 1964)	Lack of T and B lymphocytes	Multiple viral, bacterial, fungal and protozoal infections. Patients usually succumb within the first 12 months of life
Congenital thymic aplasia (DiGeorge syndrome) (DiGeorge 1968)	Thymus absent or small, T lymphocytes absent or reduced in number	Recurrent or chronic infection with viral, bacterial, fungal or protozoal pathogens
X-linked hypogammaglobulinaemia (Bruton's disease) (Bruton 1952)	Absence of B lymphocytes, Hypogammaglobulinaemia	Onset of recurrent bacterial infection following decay of transplacental maternal immunoglobulin
Chronic granulomatous disease	Defective intracellular killing of micro-organisms by phagocytes	Chronic and acute bacterial and fungal infection

B. Selected causes of acquired immunodeficiency

Protein loss	protein-losing enteropathy, nephrotic syndrome, protein hypercatabolism
Malignancy	especially Hodgkin's disease, leukaemias and myeloma
Drugs	steroids, alkylating agents (cyclophosphamide, chlorambucil), purine antagonists (6-mercaptopurine, azathioprine), cyclosporin A
Viral infection	measles, EBV, HIV
Surgery/trauma	
Malnutrition	
Ageing	

[a] Primary sources: Fudenberg et al. 1980; Webster 1983; Chapel and Haeney 1984

susceptible to mycobacterial, protozoan, fungal, viral and opportunistic bacterial infections. Defects of phagocyte function commonly present as infections caused by staphylococci, fungi and gram-negative bacteria, while some complement disorders predispose the individual to neisserial infections. Specific infections are sometimes associated with particular forms of immunodeficiency. Thus, for instance, ECHO virus encephalitis is common in hypogammaglobulinaemia and progressive Epstein-Barr virus infection in X-linked lymphoproliferative disease. Patients with immunodeficiency also exhibit an increased incidence of malignant disease. Malignancy, usually of lymphoreticular origin, has been reported in about 5–15% of patients with certain forms of immune defects (Penn 1977, 1985).

Correlation of clinical symptoms with plasma immunoglobulin concentrations or with the frequency and functional activity of T lymphocytes suggests that a relatively severe immune deficit (<60% of control values) is necessary for significant changes in the integrity of host resistance.

At present there is a lack of well-documented correlations in man between quantitative or qualitative changes in immune function and the appearance of clinical signs of immunodeficiency. It is therefore necessary to examine whether experimental animal studies provide more detailed information about immunological reserve and whether this information is of value in human risk assessment.

Experimental studies of immunotoxicity

The catalogue of chemicals which have been reported to influence the integrity of immune function is continually growing (Descotes 1988). Only a relatively small number of such chemicals have, however, been the subject of exhaustive investigation. To illustrate the issues raised in this review it is necessary only to consider examples from each of three chemical classes which have received more attention than most; the polyhalogenated hydrocarbons, the organotins and heavy metals.

Polyhalogenated hydrocarbons. A number of polyhalogenated hydrocarbons (PHH) have inadvertently become widespread environmental pollutants and their inherent lipophilicity and low biodegradability result in their concentration within the food chain, including human milk. There are numerous reports that chemicals within this group, notably the polychlorinated biphenyls (PCB) (Vos and Van Driel-Grootenhuis 1972; Loose et al. 1978; Thomas and Hindsill 1978) the polybrominated biphenyls (PBB) (Luster et al. 1978b, 1980; Fraker 1979) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Vos et al. 1973, 1978; Sharma et al. 1978; Hindsill et al. 1980) are, under the appropriate conditions of dose and exposure, associated with immunotoxicity in experimental animals. Clark et al. (1981, 1983) have reported that in mice even a cumulative dose of as low as 4 ng/kg TCDD (weekly injections of 1 ng/kg over a 4-week period) results in a significant impairment of the generation of cytotoxic T lymphocytes in response to allogeneic cells. If confirmed, this finding will be of significance in determining accurate no observed effect levels.

A common feature of exposure to immunotoxic PHH is long-lasting thymic atrophy (Kerkvliet 1984). In addition, there is mounting evidence for a causal relationship between the molecular structure of PHH, activation of an aromatic hydrocarbon gene complex and immunotoxic activity. The aromatic hydrocarbon (Ah) gene complex in mice is inherited as an autosomal dominant trait (Nebert et al. 1972) and controls responsiveness to induction of cytochrome P-448-dependent enzymes, of which aryl hydrocarbon hydroxylase (AHH) has been most extensively studied. A putative gene product of the Ah gene complex is a cytosolic receptor protein which binds stereospecifically with certain PHH congeners (Poland et al. 1976). It is currently believed that in susceptible strains, following binding, the receptor-ligand complex translocates to the nucleus (Greenlee and Poland 1979; Okey et al. 1979, 1980), interacts with DNA (Carlstedt-Duke et al. 1981) and results in gene activation and the induction of monooxygenase activity in sensitive tissues, including the thymus.

Experiments with hybrids and backcrosses have shown that TCDD-mediated immunosuppression correlates closely with AHH inducibility (Silkworth and Vecchi 1985). Furthermore, investigations with a series of PCB isomers have demonstrated that immunotoxic effects were found in C57BL/6J (Ah^b) but not DBA/2J (Ah^d) mice (Silkworth and Vecchi 1985). In most cases the immunotoxicity of PHH is associated with thymic atrophy in addition to AHH induction. However, functional immunosuppression can occur in the absence of morphological changes in the thymus (Silkworth and Vecchi 1985). Collectively, these data suggest that PHH-mediated effects on the immune system are related to the Ah gene complex and the induction of microsomal enzymes. A critical lesion in the immune system has yet to be defined and it is worth emphasising that reduced thymic cellularity and metabolic alterations following induction of hepatic and extra-hepatic enzymes may play roles of some importance in effecting changes in immune status.

While there is compelling evidence that TCDD and other halogenated aromatic hydrocarbons are immunotoxic in rodents, the majority of data available from the examination of dioxin-exposed individuals might be interpreted to suggest that man is less susceptible to TCDD-induced changes in immune competence (Reggiani 1978; Knutsen 1984). Such an assumption is not unreasonable, as extensive inter-species differences in susceptibility to TCDD-induced toxicity have been reported. A cautionary note is, however, appropriate as the route and level of exposure to TCDD in subjects studied immunologically was not determined. Moreover, studies of rats and mice have revealed that the developing immune system of the foetus and newborn is particularly susceptible to the immunotoxic effects of TCDD (Vos and Moore 1974).

In the case of polybrominated biphenyls, however, there are some reports that exposure is associated with measurable changes in human lymphocyte function (Bekesi et al. 1978, 1983). Also, there exists evidence that individuals exposed to polychlorinated biphenyls through consumption of contaminated rice oil exhibit various immunological abnormalities (Chang et al. 1981, 1982a, b; Lee and Chang 1985) and an increased susceptibility to respiratory infection (Shigematsu et al. 1978).

Organotins. The long chain dialkyltin compounds, in particular di-*n*-butyl (DBTC) up to di-*n*-octyltin dichloride (DOTC), have been shown to cause thymic atrophy (Seinen and Willems 1976; Seinen et al. 1977a; Miller et

al. 1984) and immune dysfunction (Seinen et al. 1977b; 1979). Although the mechanism(s) through which dialkyltin compounds influence the thymus are not fully understood, it has been possible to exclude indirect effects resulting from either stress-related increases in corticosteroids (Seinen and Willems 1976) or diminished somatotrophic hormone release (Penninks and Seinen 1985). The suggestion is that dialkyltins exert a direct anti-proliferative effect on lymphocytes; a view supported by in vitro studies (Penninks and Seinen 1987). To our knowledge the influence of organotins on the human immune system has not, as yet, been studied.

Heavy metals. In recent years there has been an increasing interest in the possible immunotoxic properties of heavy metals. The most thoroughly studied metal in this respect is lead. Since the first reports implicating lead as an immunotoxin (Koller 1973; Koller and Kovacic 1974) there have been a number of confirmatory studies (Koller et al. 1976; Luster et al. 1978a; Gaworski and Sharma 1978; Faith et al. 1979; Neilan et al. 1983). In fact it has been suggested that exposure of rodents to lead may result in an impaired capacity to resist bacterial or viral challenge (Hemphill et al. 1972; Gainer 1977). It is worth emphasising, however, that in some rodent studies lead was found to have no influence on (Lawrence 1981; Kimber et al. 1986a), or to enhance (Kerkvliet and Baccher-Steppan 1982), various immune functions. Preliminary results suggest that lead fails to markedly influence immune activity in man. Thus, Kimber et al. (1986b) observed that individuals chronically exposed to inorganic lead, and who exhibited blood lead levels comparable to those previously associated with immunotoxicity in rodents, possessed normal cellular immune function and plasma immunoglobulin concentrations.

Host resistance models

Host resistance models in rodents presently provide the only sure method for examining the influence of xenobiotics on the functional integrity of the immune system and its ability to efficiently eliminate pathogenic micro-organisms and tumour cells. The development of such models also provides an opportunity to assess directly the functional reserve of the immune system. It is apparent from clinical data and experimental studies that impairment of particular components of the immune system is characteristically associated with increased susceptibility to different types of infectious disease. Thus, for instance, challenge of mice with *Listeria monocytogenes* has been found to provide a reproducible method for detecting altered T lymphocyte or macrophage function (Dean et al. 1980; Vos et al. 1984). In contrast, resistance to certain transplantable tumour cells has been correlated with natural killer (NK) cell function (Dean et al. 1987).

The preliminary data available suggest that, although there is some variation between resistance models, the immune system has a significant functional reserve which must be overcome before changes in susceptibility to infection or tumour development are observed (Dean et al. 1987). Clearly there is a need for more detailed studies to provide the information required for accurate interpretation of immune function studies and assessment of toxicological significance. A cautionary note is necessary, however, as the functional reserve of the immune system may itself be variable. Many factors, including age and nutritional status, have considerable influence on immunological competence and insults to the immune system which are of little clinical significance in the young, healthy adult may be of greater importance in infants, the aged or the chronically sick.

Adverse immune reactions induced by chemicals

The directly immunosuppressive effects of xenobiotics or their metabolites have to be distinguished from their sensitising effects which may lead to *autoimmunity* and *allergy*. As reviewed above, apart from the intentional immunosuppression caused by immunomodulatory drugs, at present there is little clear evidence, albeit much concern, that xenobiotics cause unintended functional immunosuppression in man. By contrast, allergy and autoimmunity are well documented, frequent and often serious events in humans exposed to chemicals, especially drugs (DeSwarte 1986).

Autoimmunity versus allergy

Although the present report is confined to chemically induced autoimmunity, it should be emphasised that the latter shares a number of features with allergic responses to chemicals. Firstly, in both allergy and autoimmunity the immune system is stimulated to specific responses that are harmful to the body. Secondly, there are very strong effects of genetic factors predisposing to both allergic and autoimmune reactions to chemicals. In genetically susceptible individuals, even trace amounts of a chemical can elicit an adverse immunological response, whereas genetically resistant individuals will tolerate much higher doses of the chemical without showing any adverse effects. Often, these genetic effects are so strong that, when studying a mixed population consisting of susceptible and resistant individuals, the false impression may arise that there are no dose-effect relationships as far as allergic and autoimmune reactions to a chemical are concerned. Such relationships do become evident, however, when the susceptible population alone is studied.

Allergic and autoimmune reactions to chemicals can be *distinguished* as follows: in allergy, the adverse immune response is restricted to the offending exogenous agent present in the tissue. In chemically-induced autoimmunity, by contrast, the adverse immune response is not restricted to the chemical compound inducing it, but involves responses to self-antigens as well. If the inducing agent is a nonspecific immunostimulator, the adverse immune response may not be directed toward the inducing agent at all, but be confined to anti-self responses.

Classification of adverse immune reactions by their effector mechanisms

In 1963, Gell and Coombs classified adverse immune effector mechanisms into four basic types; these are shown in Table 2, together with some modifications and additions. While Table 2 only gives examples of adverse immune reactions, and only those induced by chemicals, it should be realised that protective immune reactions, e.g. those directed against infectious agents, use exactly the same effector mechanisms. With the exception of type IV

Table 2. Classification of the effector mechanisms of adverse immune reactions[a]

Gell and Coombs 1963	Immune effector mechanisms		
	Designation	Principal components	Examples of harmful tissue reactions
–	Neutralisation	Antibody	Insulin resistance, pernicious anaemia, myasthenia gravis
Type I	Anaphylactic, reaginic, immediate-type hypersensitivity	IgE antibody, mediators released from mast cells	Asthma, urticaria, allergic shock, hay fever
Type II	Cytotoxic	Antibody	Haemolysis, leukopenia, thrombocytopenia[b]
Type III	Immune complex	Antigen-antibody complexes	Vasculitis, glomerulonephritis, serum sickness, SLE
Type IV	Delayed hypersensitivity	T cells and macrophages	Contact dermatitis, berylliosis

[a] Taken from Sell (1987) and modified
[b] There is evidence that in certain patients the immunologically mediated cytopenias are mediated by excessive activity of T suppressor cells rather than antibody

reactions, all the effector mechanisms listed in Table 2 are antibody mediated. It can hardly be overemphasised, however, that in the vast majority of cases it is the T lymphocyte that determines whether or not antibody is produced.

Definition and classification of autoimmune diseases

Autoantibodies, the products of autoreactive B lymphocytes, occur in most of the autoimmune diseases (Table 3). In some of these conditions it is the autoantibodies themselves that directly cause the pathological changes. Examples here are the autoantibodies directed against the acetylcholine receptor which cause myasthenia gravis, those directed against red cells which cause autoimmune haemolytic anaemia, and those directed against the glomerular basement membrane which cause Goodpasture's syndrome. In other autoimmune diseases, however, autoantibodies are associated with the disease and indicate pathological damage, but alone may fail to initiate the disease process. Instead, the initial lesion may be partly or entirely caused by cellular immune mechanisms. Examples of the latter are Hashimoto's thyroiditis which is associated with autoantibodies against thyroglobulin and other thyroid antigens, and juvenile diabetes mellitus which is associated with autoantibodies against a cytoplasmic component of pancreatic islet cells. Thus, the appearance of serum autoantibody does not necessarily imply the existence of autoimmune *disease*. For instance, antinuclear autoantibodies occur in a proportion of healthy middle-aged women, and, with aging, rheumatoid factors (autoantibodies to immunoglobulin) as well as antinuclear autoantibodies occur with increasing frequency without a concomitant autoimmune disease. In these cases, however, the autoantibodies usually are of low titre and, probably, low affinity. On the other hand, autoantibodies may be the first indicators of an autoimmune disease that becomes overt only months or years thereafter.

A convenient, albeit somewhat arbitrary, classification of autoimmune diseases divides these into organ-specific and non-organ-specific or systemic ones. *Organ-specific* autoimmune diseases are limited to a single organ, e.g. the thyroid gland in autoimmune thyroiditis, and correspondingly, the autoantibodies in these conditions are directed

Table 3. Survey of human autoimmune diseases

A. Diseases in which pathogenic autoimmune reactions are certain or likely because the self-antigens involved have been relatively well defined

Disease	Self-antigens (as defined by the autoantibodies involved)
Autoimmune chronic active hepatitis, virus-negative	Membrane and microsomes of liver cells including P-450 cytochrome isoenzymes
Autoimmune haemolytic anaemia	Membrane components of erythrocytes
Bullous pemphigoid	Basement membrane of skin
Goodpasture's syndrome (glomerulonephritis and alveolitis with linear immunoglobulin deposits along the glomerular and alveolar basement membranes)	Components of the glomerular basement membrane (GBM) and alveolar BM
Guillain-Barré syndrome	Myelin and other components of the sheets of peripheral nerves
Hashimoto's thyroiditis	Cytoplasmic or microsomal thyroid antigen, thyroglobulin
Idiopathic leucocytopenia	Membrane components of leucocytes
Idiopathic thrombocytopenia	Membrane components of platelets
Male infertility (certain cases)	Spermatozoa
Myasthenia gravis	Acetylcholine receptor at the neuromuscular synapsis
Pemphigus vulgaris	Desmosomes linking epithelial cells of the skin
Pernicious anaemia	Intrinsic factor (produced by parietal cells for absorption of vitamin B_{12})
Primary Addison's disease	Microsomal antigens in the adrenal cortex
Progressive systemic sclerosis (scleroderma)	Various antigens in cell nuclei, especially nucleoli
Systemic lupus erythematosus (SLE)	Various nuclear antigens, especially double-stranded DNA; antigens on leucocytes and erythrocytes
Thyrotoxicosis	TSH receptors
Wegener's granulomatosis (inflammatory disease of veins and arteries, especially in the lung and kidneys)	Alkaline phosphatase-like material on endothelial cells and neutrophils (Lockwood et al. 1987)

Table 3. (Continued)

B. Diseases with immunological pathogenesis in which, however, the relevant self-antigens are less well defined or unknown (in the latter case it cannot be excluded that the relevant antigens are foreign structures implanted into the tissue)

Disease	Suspected self-antigens
Glomerulonephritis with granular deposits of immunoglobulin along the glomerular basement membrane (GBM)	1) Antigens from the circulation, such as DNA, which have been implanted along the GBM 2) Autochthonous membrane structures on the epithelial cells covering the GBM, e.g. glycoproteins belonging to the gp 330 family
Juvenile diabetes mellitus (type I diabetes)	Antigens of the insulin-producing beta cells in the pancreas
Rheumatoid arthritis	Antigens of articular cartilage, such as chondroitin sulphate and a core protein of proteoglycan: collagen; IgG
Morbus Sjögren (lymphocytic infiltration of salivary and lacrimary glands)	Unknown
Polymyositis (lymphocytic infiltration of striated muscles)	Unknown
Dermatomyositis	Unknown
Lichen ruber	Unknown
Uveitis, certain types	Unknown antigens of the ocular lens
Primary biliary cirrhosis	Inner mitochondrial membrane
Ulcerative colitis	Unknown
Multiple sclerosis	Unknown

towards tissue-specific antigens, such as thyroglobulin. *Non-organ-specific* autoimmune diseases, by contrast, involve autoantibodies to ubiquitous self-antigens and, in a varied pattern of expression, affect several organs. The autoantibodies most frequently looked for in the laboratory investigation of these patients are antinuclear antibodies and rheumatoid factors. An example of a non-organ-specific autoimmune disease is systemic lupus erythematosus (SLE) in which high titres of IgG autoantibodies to a variety of nuclear antigens, in particular double-stranded DNA, are produced; this results in the formation of immune complexes which are deposited in the vessel walls and along basement membranes of different tissues and thus cause pathological alterations. Other examples of non-organ-specific autoimmune diseases are rheumatoid arthritis, Sjögren's syndrome, dermatomyositis, and scleroderma (progressive systemic sclerosis). Histologically, all these diseases are characterised by inflammatory lesions of the blood vessels and fibrinoid degeneration in the connective tissue; therefore, this family of diseases is also termed collagen-vascular diseases.

A given patient may have more than one autoimmune disease. Such an overlap is seen rather often among the organ-specific diseases on the one hand and among the non-organ-specific diseases on the other hand. There is hardly any overlap, however, between the organ-specific and the non-organ-specific groups of autoimmune disease, which suggests a difference in their pathogenesis (cf Fig. 2 *A* and *B*).

Role of T cells and molecules of the major histocompatibility complex (MHC) in the immune response

T lymphocytes play a central regulatory role in the immune response to both foreign antigens and self-antigens. T cells can be divided into at least two functional subclasses, T helper/inducer (Th) and T killer/suppressor cells. T cells recognise antigen via a specific two-chain receptor on their surface.

Upon stimulation by specific antigen, Th cells secrete various interleukins, which act on other cell types and induce them to perform their specific functions. One of the interleukins produced by Th cells is interleukin 2 (IL-2) which acts mainly on other T cells, especially T killer cells. Another, interleukin 4 (IL-4), induces proliferation of haemopoietic cells, mast cells and B cells; in addition, IL-4 enables B cells to switch from the production of the relatively T cell-independent IgM antibody isotype to that of antibodies of the IgG and IgE isotype. Th cells also play a central role in the development of cellular immune (type IV) reactions (Table 4C). T killer cells, by contrast, are effector cells themselves in that they directly kill target cells carrying foreign antigen on their surface.

Unlike B cells which by their specific immunoglobulin receptors can recognise and bind soluble antigen, T cells can recognise antigen only if it is presented to them on the surface of another cell in conjunction with structures of the MHC. While T killer cells recognise antigen in conjunction with class-I MHC structures, Th cells recognise antigen in conjunction with class-II MHC structures. Small chemicals, such as trinitrophenyl (TNP), can be directly coupled by covalent bonds to the cell membrane and thus be recognised by specific T cells. It is not clear whether chemicals have to directly bind to MHC structures in order to become immunogenic for T cells.

In both man and laboratory animals there is a tremendous genetic polymorphism of the MHC. A functional consequence of this polymorphism is that a given antigen A may adequately be presented to T cells by the product of an assumed MHC allele^h, but inadequately by that of an assumed MHC allele^l. Therefore, individuals possessing MHC alleles^h are high responders with respect to antigen A, whereas individuals possessing MHC alleles^l are low responders. However, an MHC high responder to antigen A may well be a low responder to the structurally different antigen B, and vice versa. In other words, the action of MHC structures is selective. The human MHC, HLA, codes for the class-I molecules A, B and C and the class-II molecules DR, DP and DQ. The products of different HLA alleles are designated by numbers, e.g. *HLA DR3.*

Role of T cells, B cells and MHC molecules in autoimmunity

During ontogeny the immune system acquires tolerance to self-antigens while developing the capacity to defend the body against foreign antigens. This distinction between "self" and "non-self" is an unique feature of the immune system and is crucial for the maintenance of health. Self-tolerance does not imply, however, that potentially auto-

Table 4. Examples of adverse immunological side-effects of drugs in man[a, b]

A. Drug-induced autoantibodies	
Disease	Inducing drug
Autoimmune chronic active hepatitis, virus-negative	Halothane, tienilic acid
Autoimmune haemolytic anaemia, certain types	α-Methyldopa, L-dopa, captopril, cefalexin, mefenamic acid, penicillins
Goodpasture's syndrome	D-penicillamine
Granulocytopenia, certain types	Aminopyrine, captopril, cefalexin, chloral hydrate, chlordiazepoxide, chlorpromazine, chlorpropamide, gold salts, mercurial diuretics, indomethacin, p-aminosalicylic acid, penicillins, sulphapyridine/sulphathiazol, thiouracils, tolazoline
Myasthenia gravis	D-penicillamine, possibly gold salts
Phemphigus vulgaris	D-penicillamine
Bullous pemphigoid	D-penicillamine
SLE	Gold salts, griseofulvin, hydralazine, phenytoin, practolol, D-penicillamine, procainamide, thiouracil, and others
Immune complex type glomerulonephritis	Gold salts, D-penicillamine and other drugs with an -SH group
B. Drug-induced immunological diseases with unknown pathogenesis	
Aplastic anaemia, certain types	D-penicillamine, phenytoin, quinacrine, oxyphenyl, phenylbutazone
Intrahepatic cholestasis/ cholangitis	Chlorpromazine, chlorpropamide, erythromycin estolate, imipramine, nalidixic acid, nitrofurantoin
Hepatitis, non-viral	Aminosalicylic acid, amiodarone, captopril, isoniazid, phenytoin and other hydantoins, and others
Hypogammaglobulinaemia	Gold salts, phenytoin
Infectious mononucleosis-like syndrome	Aminosalicylic acid, dapsone, phenytoin
Interstitial nephritis	Azathioprine, cephalosporins, furosemide, penicillins (esp. methicillin), phenindione, phenytoin, rifampicin, sulfinpyrazone, sulphonamides, thiazides, thiouracil
Lymphadenopathy/(pseudo) lymphoma/M. Hodgkin	Phenytoin and other hydantoins, possibly gold salts
Peripheral neuritis	Colchicine, gold salts, nitrofurantoin, sulfonamides
Serum sickness	Penicillins, cephalosporins, streptomycin, sulphonamides, and others
Skin: immunological drug reactions can mimic virtually *all* clinical and histological patterns of disease	Antibiotics, barbiturates, diuretics, gold salts, hydantoins, tranquilisers, and many others
Thrombocytopenia, certain types	Acetazolamide, acetylsalicylic acid, carbamazepine, cephalothin, chloramphenicol, digitoxin, gold salts, imipramine, levodopa, meprobamate, methyldopa, para-aminosalicylic acid, phenylbutanzone, phenytoin, quinidine, quinine, rifampicin, spironolactone, stibophen, sulphonamides, sulphonylureas, thiazides
Vasculitis, different types	Allopurinol, busulfan, indomethacin, isoniazid, iodides, penicillin, phenothiazines, phenylbutazone, tetracyclines, thiazides, thiouracils
C. Examples of allergic reactions to chemicals (only reactions against non-self antigens are involved)	
Allergic asthma and related conditions	Different types of allergen inhaled at the work place, such as the dust of manufactured antibiotics, ethylenediamine, formaldehyde, insecticides, isocyanates, salts of the heavy metals chromium, cobalt, mercury, nickel, platinum
Contact dermatitis	1) Very many topically applied drugs, such as antibiotics, antihistamines, local anaesthetics 2) A great variety of other chemicals, including many different organic compounds and the salts of heavy metals, such as chromium, cobalt, mercury, and nickel
Food allergy	Many different types of food additive, chemical contaminations of food

[a] Compounds are listed alphabetically and not according to the frequency of adverse immunological side-effects they induce
[b] Major sources of reference: Aaronson (1980), DeSwarte (1980), Parker (1980)

active B cells and T lymphocytes are all absent from the body. Instead, autoreactive B cells (Dighiero et al. 1983) and T cells (Cohen 1986) do occur in normal individuals, but they are held in check by mechanisms that are incompletely understood. Self-tolerance at the level of T cells, however, appears to be much tighter than at the level of B cells; this is important with respect to the T-cell by-pass concept of autoantibody formation (see below). Moreover, while the existence in normal individuals of B cells capable of producing autoantibodies to self-antigens circulating at low concentration, such as DNA, has unequivocally been demonstrated (Dighiero et al. 1983), it is not clear whether this is true for all types of self-antigen, e.g. those circulating at high concentration, such as serum albumin. In normal individuals, potentially autoreactive lymphocytes are completely or largely inactive, but under certain

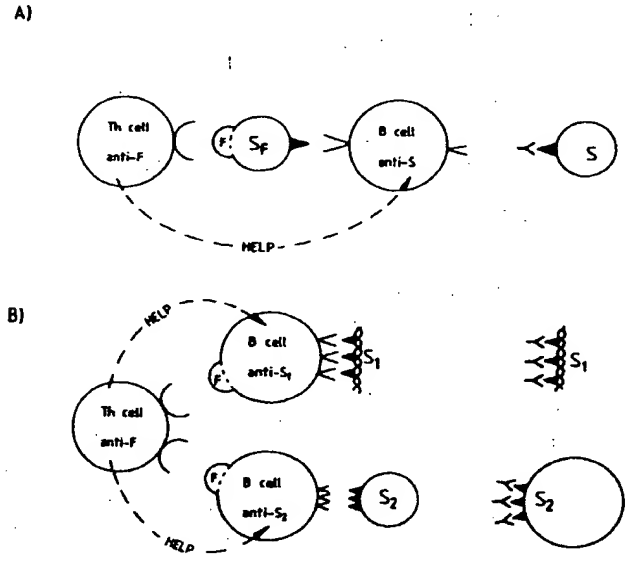


Fig. 2. Depicting two different pathways leading to autoantibody formation according to the T cell by-pass concept (Allison et al. 1971). A common feature of both possibilities is that normally occurring autoreactive B cells are stimulated to antibody formation by the *combined action* of 1) their recognition of epitopes on self-antigen and 2) the activation of adjacent Th cells. Activation of adjacent Th cells is due to the fact that a foreign antigenic determinant (*F*) has been introduced into the system. Hence, the Th cells cooperating with the autoreactive B cells react, not with native self-antigen, but with the foreign determinant F. In A, the foreign determinant F is located on the same molcule or cell as the unaltered self-epitope to which the autoreactive B cell reacts. In B, the foreign determinant F is physically separate from the unaltered self-antigen S to which the B cell reacts. As a result of this, only those of the autoreactive B cells are activated that react to self-antigens possessing repeating identical epitopes, such as cell-membrane epitopes and DNA. This hypothesis is based on the graft-versus-host (GVH) model of SLE-like autoimmune disease and has been elaborated in detail elsewhere (Gleichmann et al. 1984; Gleichmann and Gleichmann 1987). *F*: Foreign determinant; *S*: unaltered self-antigen; S_F: altered self-antigen; $\prec$: autoantibdoy

circumstances they may become activated and produce autoimmune disease. The circumstances under which this can occur and the cellular and genetic factors involved have only partially been unravelled, however. Moreover, the factors that determine whether an autoimmune disease will subside or continue are even less well defined.

The rules that govern the physiological immune responses to non-self antigens appear to be identical with those that govern pathological immune responses to self-antigens. For instance, autoreactive Th cells, too, can recognise the respective self-antigen only in association with class-II MHC structures (Londei et al. 1985; Hohlfeld et al. 1986). Furthermore, due to the absence of functionally autoreactive Th cells in healthy individuals, autoreactive B cells are unable to produce autoantibodies, or at most, produce a limited amount of autoantibody of the IgM isotype (Dighiero et al. 1983). Autoreactive B cell clones will expand and switch to the production of pathogenic IgG autoantibodies, however, whenever T cell help becomes available to them. Because the interleukins secreted by Th cells are not antigen – specific, any Th cell, irrespective of its specificity, can, in principle, provide help to an autoreactive B cell. A prerequisite for this is that functionally active Th cells come into the vicinity of the autoreactive B cell. If this requirement is fulfilled the lack of functionally autoreactive Th cells is *by-passed* and the autoreactive B cell activated.

According to the *T cell by-pass concept* (Allison et al. 1971), the lack of functionally autoreactive Th cells can be overcome in two different ways, as depicted in Fig. 2. First, a self-antigen, such as thyroglobulin, might be chemically altered so that specific Th cells react to the new antigenic determinant, or carrier, while autoreactive B cells react to the unaltered part, or hapten, and start to produce autoantibodies to it (Weigle 1980). In this case, a carrier-hapten bridge brings specific Th and B cells together and the specificity of the resulting autoantibody formation is restricted to the particular self antigen which was altered (Fig. 2 *A*). This might explain formation of organ-specific autoantibodies. Second, chemicals may alter B cells directly, including autoreactive B cells. Th cells will then provide help to all B cells, irrespective of the specificity of the B cells. There is no carrier-hapten bridge in this case, but B cells have to react to self-antigen that is physically separate from what Th cells recognise (Fig. 2 *B*). As a consequence, the B cells which have a selective "advantage" might be those specific for self-antigens possessing repeating identical epitopes on a rigid backbone, and hence can undergo multipoint high-avidity binding and cross-link the Ig receptors on the B cell. This would explain the formation of non-organ-specific autoantibodies, such as anti-DNA, as opposed to formation of antibodies against self-antigens not possessing repeating identical epitopes on a rigid backbone, such as thyroglobulin (Gleichmann et al. 1984; Gleichmann and Gleichmann 1987).

Many autoimmune diseases show statistically significant associations with certain HLA alleles, i.e. the statistical chance of individuals possessing certain HLA alleles to develop a certain autoimmune disease is greater than that of individuals possessing different HLA alleles. As opposed to such positive HLA associations, there are also negative HLA associations where possession of a certain HLA allele apparently protects the carrier from developing a certain autoimmune disease. This is also true for chemically induced autoimmune diseases (Table 5). The exact role of HLA structures in the pathogenesis of chemically induced autoimmune diseases is, however, not known.

Theoretically, there are several possibilities, which are not mutually exclusive, to account for these associations. One of these is that chemicals alter certain self-antigens so that T cells recognise them as foreign. Thus, one may speculate that D-penicillamine directly alters the acetylcholine receptor (cf Tables 3 and 4, Fig. 2 *A*). Self-antigen thus rendered foreign would then be presented to Th cells by MHC class-II structures, a function which the products of certain MHC class-II alleles are more apt to perform than others. Another possibility is that chemicals directly alter MHC class-II structures on B cells and thus render them foreign for Th cells (Fig. 2 *B*). Consistent with this hypothesis is the fact that B lymphocytes which were experimentally rendered "foreign" by the coupling of TNP to their membrane elicited GVH-like reactions by normal syngeneic Th cells and, as a consequence, showed an en-

Table 5. Selected examples of HLA phenotypes and other genetically determined traits that predispose to chemically induced autoimmune diseases in man

Disease	Aetiologic agent	Predisposing genetic factors		Authors
		HLA	Other	
Myasthenia gravis	D-penicillamine (antirheumatic drug)	DR1, Bw35	slow sulphoxidisers	Dawkins et al. 1982; Emery et al. 1984; Panai et al. 1983
Glomerulonephritis due to granular IgG deposits at the basement membrane, proteinuria	D-penicillamine	DR3, DR4		Dawkins et al. 1982; Wooley et al. 1980
Glomerulonephritis due to granular IgG deposits, proteinuria	gold sodium thiomalate (antirheumatic drug)	DR3, B8*	slow sulphoxidisers	Wooley et al. 1980 Perrier et al. 1985; Hakala et al. 1986
Autoimmune thrombocytopenia	gold sodium thiomalate	DR3		Coblyn et al. 1981; Adachi et al. 1984
Drug-induced SLE	hydralazine (anthihypertensive drug)	DR4	slow acetylators, female sex	Batchelor et al. 1980
Scleroderma-like lesions (sclerosis of the skin, Raynaud's phenomenon, arthralgia and arthritis, pulmonary and portal fibrosis, thrombocytopenia)	vinyl chloride (industrial chemical)	DR5		Black et al. 1986

* While HLA DR3 and DR8 are susceptibility factors, DR7 determines resistance

hanced production in vivo of IgG antibodies (Ptak et al. 1984). Conceivably, some compounds producing autoimmunity bind to certain class-II structures better than others. Evidence for a preferential interaction of certain drugs with certain HLA class-I structures has been obtained by Claas and van Rood (1985). These authors incubated human peripheral blood leucocytes with high concentrations of various drugs in vitro and demonstrated consistent patterns of selective blocking of certain HLA class-I structures by certain drugs.

In the experiment of Ptak et al. (1984) mentioned above, murine B cells were rendered "foreign" by TNBSA (trinitrobenzene sulfonic acid), a chemical that covalently binds to protein. Whether other chemicals, such as drugs, will spontaneously bind to the membrane of lymphoid cells in vivo and thus render them "foreign" is largely unknown. Some indirect evidence that this may, indeed, happen has been obtained from studies on the sensitization to penicillin in humans (de Weck 1983) and D-penicillamine in mice (Nagata et al. 1986). In the latter case, it was shown that even after oral application of a high dose of D-penicillamine, peritoneal macrophages of recipient mice were altered in such a way that they elicited reactions by specific Th cells (Vogeler and Gleichmann 1988). Whether this alteration is, indeed, responsible for the SLE-like autoimmune disease inducible by D-penicillamine in the rat (Donker et al. 1984) and for human autoimmune diseases induced by this drug (Table 4) is yet unknown.

Cells whose function includes presentation of antigen to Th cells constitutively express class-II MHC structures. Such cells are dendritic cells, Langerhans cells of the skin, and B lymphocytes, especially activated B cells. Many other cell types, however, including, for instance, epidermal cells, thyrocytes and pancreatic islet cells, facultatively express class-II structures during inflammatory responses in the respective tissue. This is due to the local production of immunological mediators, such as tumour necrosis factor, gamma-interferon and lymphotoxin. Inflammatory reactions also include destruction of cells and hence release and/or alteration of self-antigens. These two phenomena together enhance the chance for (self-) antigen to be recognised by (autoreactive) Th cells and thus could increase the chance for development of autoimmune disease (Londei et al. 1985; Pujol Borell et al. 1987). With respect to chemically-induced autoimmunity, it would be of value to examine whether chemicals can induce the expression of class-II MHC genes.

Chemicals as aetiological agents of human autoimmune diseases

In contrast to the pathogenesis of autoimmune diseases which begins to be unravelled, as outlined above, almost nothing is known about their *aetiology*. In those admittedly few cases, however, where an aetiological agent of human autoimmune disease can be identified, most often this agent is a chemical compound, and this, in turn, most often is a drug. For three reasons it is not legitimate in the present state of ignorance to draw the converse conclusion from these observations, namely that chemicals are also the main suspects for the many cases of autoimmune disease with unknown aetiology. Firstly, there may be an observer's bias for drug-induced autoimmune diseases in that patients receiving drugs are under close medical supervision to begin with, so that chances for such cases being noticed and reported are presumably greater than with other aetiological agents. Secondly, the natural history of autoimmune diseases induced by drugs is often different from that of the same disease developing spontaneously, because drug-induced autoimmune symptoms usually disappear after withdrawal of the drug, whereas idiopathic autoimmune diseases often progress or follow a course characterised by relapses and remissions. Thirdly, it is conceivable that autoimmune diseases develop without an exogenous cause due to spontaneously arising errors in the regulation of the immune system. This possibility is sup-

ported by the fact that there are inbred strains of animals that spontaneously develop autoimmune disease.

As can be seen from Table 4, drugs can induce a great variety of organ-specific and non-organ-specific autoimmune diseases. Several drugs can induce more than one kind of autoimmune disease (see, for instance, D-penicillamine and phenytoin), and certain drugs, such as penicillin, can induce both autoimmune disease and allergy (Table 4). With the exception of adverse immunologic reactions to penicillin (de Weck et al. 1983), only very few drug reactions have been studied in a systematic fashion, however. In particular, investigations of specific anti-drug reactions of T lymphocytes are scarce. Hence, in most instances of drug-induced autoimmunity it has not been proven if, indeed, Th are involved in the pathogenesis, as one would postulate on theoretical grounds. (A reaction of Th cells against the drug does not preclude, of course, that antibodies to that drug may also be produced.) It is also unclear, in the vast majority of cases, whether an adverse immunological reaction to a given drug is elicited by the compound or a metabolite.

A provocative, albeit indirect, observation for the involvement of drug metabolites in human autoimmune disease has recently been reported by Beaune et al. (1987). They showed that patients who developed a nonviral hepatitis after treatment with tienilic acid had IgG autoantibodies directed against the isoform P-450-8 of cytochrome P-450 obtained from human liver microsomes. These autoantibodies specifically inhibited the hydroxylation of tienilic acid by human liver microsomes. The authors suggest that cytochrome P-450, originally present in the endoplasmic reticulum of the hepatocyte, could be alkylated by a reactive metabolite and migrate onto the hepatocyte membrane surface. At this level, the modified protein could be recognised by specific Th cells reacting to that part of the molecule derived from the reactive metabolite. These functionally active Th cells, in turn, would allow formation of IgG autoantibodies that recognise the native protein (cf Fig. 2 *A*). One might add that, in retrospect, formation of autoantibodies to cytochrome P-450 is perhaps not totally surprising because this enzyme system generates highly reactive metabolites from a variety of different parent compounds. Prior to metabolic transformation, the compounds usually are not very reactive and, hence, not, or hardly, immunogenic. Thus, in an immunological sense this particular enzyme system marks a strategic borderline between self and non-self.

Genetic factors predisposing to human autoimmune diseases induced by drugs or occupational chemicals

It is clear from the few studies performed that both immunological and pharmacological genetic traits can be involved in the development of drug-induced autoimmunity in man. As an immunogenetic factor, the HLA alleles of the respective patients were determined, while the pharmacogenetic traits studied were those that are relevant for the metabolism of a particular drug (Table 5).

In contrast to the autoimmunising side-effects of drugs which are well documented, little is known about the autoimmunising potential of occupational and environmental chemicals. Severe scleroderma-like lesions have been reported in workers exposed to vinyl chloride (Table 5) as well as workers exposed to quartz (Ziegler et al. 1986). Moreover, scleroderma-like lesions, but also SLE, have been reported in women carrying silicon-containing breast protheses (Kumagai et al. 1984; Guillaume et al. 1984). An SLE-like disease can also develop in humans exposed to a food additive, azodyl tartazine, or the industrial chemical hydrazin, and in monkeys and humans fed an amino acid present in alfalfa seeds (Pereyo 1980; Malinow et al. 1982; Reidenberg et al. 1983).

Mercurials as aetiological agents of autoimmunity and increased IgE production

A chemical which induces autoimmunity and has been studied in some depth is mercury. In several rodent species, mercurials have been shown to cause an SLE-like autoimmune syndrome as well as a marked increase in IgE formation. A prominent feature of the mercury-induced autoimmune syndrome in rodents is glomerulonephritis, and this disease at least has also been documented in humans exposed to mercurials (Table 6). The observations in man were made in cases of mercury poisoning or exposure to mercury as a constituent of drugs or cosmetics. In all these cases, subjects were exposed over a relatively short period of time to high concentrations of mercury. While it is unknown whether or not the concentrations of mercury existing at the work place and in the environment constitute a risk with regard to immunopathology, it is noteworthy that the immunopathological signs inducible by mercury are not confined to certain mercury compounds or routes of administration of such compounds (Table 6). Moreover, the dosages of $HgCl_2$ that induce autoimmune disease and enhanced IgE formation in rodents are clearly below the dose range in which general toxicity is observed.

The pathogenesis of mercury-induced autoimmunity has been analysed in detail in the rat. In this species, there is a stringent genetic control of $HgCl_2$-induced autoimmunity and increased IgE production which is determined by three to four independently segregating loci. One of these loci segregates with the MHC and exerts a strong effect, the others have not been identified. Of 22 inbred strains of rat studied, the Brown Norway strain was the most susceptible, because it developed all the symptoms listed in Table 6; other strains were partially susceptible, and yet others, such as Lewis, were resistant. In Lewis rats, even doses of $HgCl_2$ which induce acute tubular necrosis failed to induce autoimmune phenomena.

All autoimmune phenomena induced by $HgCl_2$ are T cell dependent, since they fail to develop in $HgCl_2$-treated athymic rats. The pathological alterations of mercury-induced autoimmunity resemble those of chronic GVH disease (GVHD) (Druet et al. 1987). Chronic GVHD is caused by an excessive activation of Th cells which, secondarily, activate other immune cells, in particular B cells; the latter then produce antibodies, especially SLE-like autoantibodies of the IgG isotype (Gleichmann et al. 1984). In mercury-induced autoimmunity, too, there is an excessive activation of Th cells. This suggests that there is a common final pathway leading to SLE-like autoimmunity in chronic GVHD and mercury-induced autoimmunity.

The popliteal lymph node assay (PLNA) in rodents, a test predicting the sensitizing and/or immunostimulatory potential of xenobiotics

The central role of T cells in the initiation of both autoimmunity and allergy has been emphasised. Hence, in the

Table 6. Survey of autoimmune and other immunopathological alterations induced by exposure to mercury compounds of humans and genetically susceptible strains of rat, respectively

Species studied	Mercury compounds studied	Route of application	Pathological alteration induced	Selected references
Man[a]	Various Hg-containing drugs and ointments	Oral, percutaneous, and various routes of injection	Membranous glomerulonephritis with granular IgG deposits in the mesangium and at the glomerular basement membrane	Fillastre et al. 1984; Tubbs et al. 1982
			Contact dermatitis and other forms of dermatitis	Taugner and Schütz 1966
			Enhanced IgE formation in vitro	Kimata et al. 1983
Rat[a, b]	$HgCl_2$, CH_3HgCl, Hg-containing drugs and ointments	Inhalative, intraperitoneal, oral, percutaneous, subcutaneous	Lymphadenopathy, splenomegaly	Druet et al. 1983, 1987
			Lymphocytic infiltration of salivary glands (similar to M. Sjögren)	Aten and Weening 1985
			Intravascular blood coagulation	Druet et al. 1983
			IgG autoantibodies against the glomerular basement membrane (similar to Goodpasture's syndrome)	Druet et al. 1983, 1987
			Membraneous glomerulonephritis with granular IgG deposits in the mesangium and at the glomerular basement membrane	Druet et al. 1983, 1987; Weening et al. 1981
			IgG deposits in the walls of blood vessels	Druet et al. 1983, 1987
			IgG autoantibodies to various nuclear antigens and other autoantibodies	Weening et al. 1981
			Polyclonal B cell stimulation	Druet et al. 1983, 1987
			Extreme increase in total serum IgE and, if antigen is administered simultaneously, formation of specific IgE antibodies	Druet et al. 1983, 1987

[a] Some of the abnormalities listed above could also be induced in rabbits and guinea pigs exposed to $HgCl_2$ (Polak et al. 1968; Albini et al. 1983)

[b] Almost all the abnormalities induced in the rat can also be induced by $HgCl_2$ administration to susceptible strains of mouse (Fleuren et al. 1985; Robinson et al. 1986; Hultman and Enestrōm 1987; Mirtschewa et al. 1987; Pietsch et al. 1987)

preclinical test phase there is a need for simple laboratory tests to predict the immunogenicity of chemicals for T cells. The PLNA in laboratory rodents might suit this purpose. The scheme of the PLNA is shown in Fig. 3.

A survey of results obtained by the PLNA is given elsewhere (Gleichmann et al. 1988; Gleichmann and Klinkhammer 1988; Kammüller and Seinen 1988; Kammüller et al. 1988; Stiller-Winkler et al. 1988). During the primary PLN response to small chemicals or conventional antigens, the maximal response in the mouse hardly exceeds a PLN weight index of 10. In the rat, however, the values of PLN indices are usually higher than in the mouse. With most chemicals tested the primary PLN response, if inducible, peaked within the first 10 days after injection and returned back to normal at week 3–4; exceptions from this rule were seen with high dosages of heavy metals ($HgCl_2$, $CdCl_2$) and, in particular, quartz.

Quartz induced an ever progressing PLN enlargement so that after 6 months the weight increase of the draining PLN had reached values of up to 200 times that of the contralateral PLN (Stark et al. 1988). Quartz also was exceptional in that the lymph node enlargement induced did not require presence of T lymphocytes (Zaidi et al. 1979); this fits the notion that quartz (SiO_2) is not an antigen. Quartz is phagocytosed by macrophages and, since it is undigestible, persistently activates these cells to release factors which, secondarily, activate lymphocytes. Thus, in the case of quartz the PLNA reflects non-specific immunostimulatory properties of the test compound.

By contrast, in all other instances where this was tested (phenytoin, D-penicillamine, streptozotocin, captopril, $AuCl_3$) the PLN response was not only T cell-dependent, but also specific. Specificity was proven by the fact that primed mice showed an enhanced PLN response upon a second injection of the same, but not another compound (Hurtenbach et al. 1987; Klinkhammer et al. 1988).

Injection of chemicals into a hind footpad is an artificial manoeuvre designed to detect the sensitizing potential of chemicals. In real life, chemicals are taken up by different routes, such as the oral and the inhalative routes, so that the mesenteric and mediastinal lymph nodes and the spleen, respectively, are the candidate lymphoid organs that may harbour sensitized T lymphocytes. It is of great practical importance, therefore, to have a simple test sys-

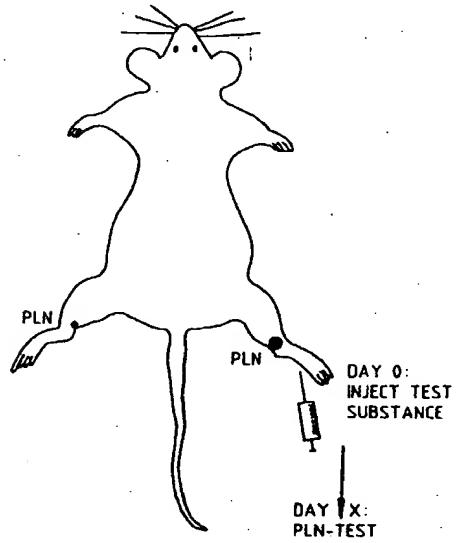


Fig. 3. Scheme of the *direct popliteal lymph node assay (PLNA)* in rodents. The test compound is injected subcutaneously without adjuvant into one hind footpad of a test animal. The contralateral side is left untreated, or inoculated with the solvent of the test compound and thus serves as an internal control. Days thereafter, an ensuing immune reaction can be assessed by removing the PLNs and determining either the PLN weight index, or, more sensitively, the number of cells in the PLNs, or [^{3}H]thymidine incorporation into the PLNs. PLN cells may also be analysed by flow cytometry

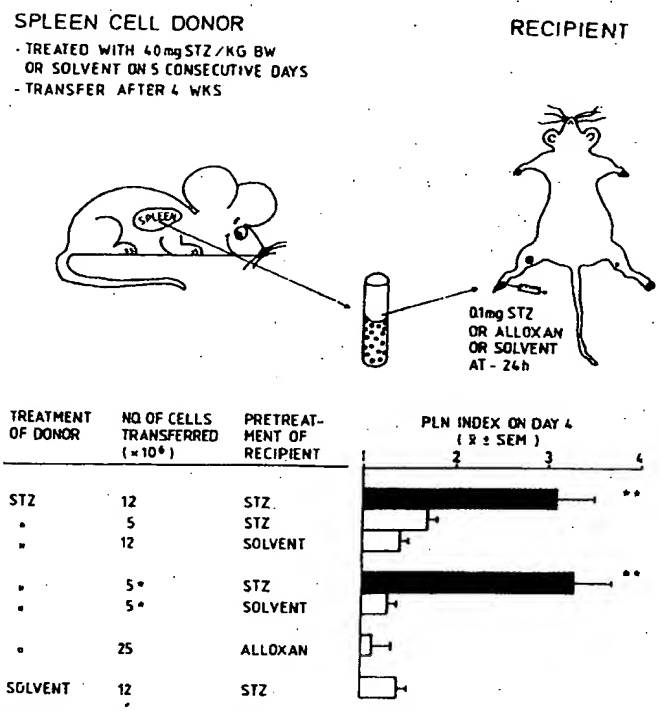


Fig. 4. Experimental design and results of the *adoptive transfer PLNA*. Mean values SEM of the PLN weight index are shown. Spleen cells of BALB/c donor mice, which had received five i.p. injections of either streptozotocin (STZ) or the solvent of STZ, were inoculated s.c. into one hind footpad of syngeneic mice. The recipients had received an s.c. injection of either a sub-immunogenic dose (0.1 mg) of STZ or alloxan or solvent into the same footpad 24 h before the cell transfer. Groups of seven mice each were used as recipients. * = enriched for T cells; ** = $p < 0.005$ versus controls. Reproduced from Klinkhammer et al. (1988) with kind permission of the authors and the American Diabetes Association Inc.

tem in which mesenteric, mediastinal or splenic T lymphocytes from rodents, which received a chemical in the context of routine toxicity testing, can be assayed for possible sensitization to that chemical. Such a test system has recently been established in the form of the adoptive transfer PLNA (Fig. 4). Potentially sensitised T cells were taken from donor mice that had received five i.p. injections of streptozotocin and had then been rested for 4 weeks. It should be noted that by histopathological criteria, the spleens of these mice were normal. Splenic T cells obtained from such donor animals were injected into the footpad of syngeneic rodents, together with a dose of streptozotocin which by itself is too low to induce a primary PLN response. Two to five days after the cell transfer, a specific PLN enlargement was seen in the recipient. In this experiment, the recipient is rather inert in as much that it serves only to properly present the test compound to the donor's T cells and reflect (by PLN enlargement) their reaction. Thus, the adoptive transfer PLNA showed that sensitized T cells from the spleen of a donor mouse, which had been exposed to a drug by multiple i.p. injections, can be demonstrated by specific restimulation in the PLNA. The optimal conditions for the adoptive transfer PLNA need to be defined. It should be tested, for instance, if a chemical will also be recognised by the transferred memory T cells if it is administered to the prospective recipient not into the footpad along with the donor T cells, but via the route and at a dosage that represent a more realistic exposure to that chemical. This should allow measurement of T cell reactions to immunologically relevant metabolites, provided these reach the circulation. It is mandatory, however, that test systems, such as the PLNA, be validated, before they can be used in routine toxicology.

Methods to detect adverse immunological reactions to chemicals in man

In man, tests to detect adverse immunologic reactions to chemicals are usually performed a posteriori, i.e. after a presumed sensitization to a xenobiotic. At the antibody level, a variety of serological tests to detect specific antibodies to the suspected drugs can be performed in vitro. In vivo, the scratch test is frequently used which involves topical application of the suspected drug into the dermis. The scratch test preferentially detects antibodies formed against a drug, in particular those of the IgE isotype (type I reaction). At the T cell level, chemical compounds are tested for their capacity to elicit contact dermatitis (type IV reaction).

All these tests actually measure allergic reactions, and they are thus not necessarily relevant to autoimmunity. As discussed above, it is likely that many chemicals known to cause autoimmune disease primarily trigger reactions by specific Th cells which secondarily activate autoreactive

cells, especially autoreactive B cells. Such a chemical need neither elicit contact dermatitis nor the formation of antibodies specific for that chemical, let alone specific IgE antibodies. What is needed, therefore, is a test method demonstrating specific sensitisation of human Th cells to small chemicals. In principle, such a sensitisation can be demonstrated by the lymphocyte transformation test in vitro, where peripheral blood lymphocytes (essentially T cells) are obtained from a sensitised patient and cultured in the presence of the suspected drug. A problem of the lymphocyte transformation test in its present form is, however, that it has produced many "false" negative, and also some "false" positive results. Nonetheless, the general opinion is that the accuracy of this test can be considerably improved by further standardization (Stejskal et al. 1986) and, in particular, elaboration of the conditions required for "presenting" the test compound to the responder T cells. Here, too, the question arises whether it is the mother compound or a metabolite that is seen by the sensitized T cells of a given patient. With certain drugs, evidence in favour of metabolites has been presented by Victorino et al. (1985) and Merk et al. (1988). While Merk and colleagues failed to detect T cell transformation in response to the mother compounds, they did observe T cell transformation in response to drugs that were pre-incubated with liver microsomes, as a source of cytochrome P-450. Further experiments of this type need to be performed.

References

Aaronson DW (1980) Asthma: general concepts. In: Patterson RW (ed) Allergic diseases. JB Lippincott Company, Philadelphia, Toronto, pp 231–278

Adachi JD, Bensen WG, Singal DP, Powers PJ (1984) Gold-induced thrombocytopenia; platelet associated IgG and HLA typing in three patients. J Rheumatol 12: 355–357

Albini B, Glurich I, Andres A (1983) Mercuric chloride-induced immunologically mediated diseases in experimental animals. In: Porter GA (ed) Nephrotoxic mechanisms of drugs and environmental toxins. Plenum Medical Books Company, New York, pp 413–423

Allison AC, Denman AM, Barnes RD (1971) Hypothesis. Cooperating and controlling functions of thymus-derived lymphocytes in relation to autoimmunity. Lancet ii: 135–140

Aten J, Bosman CB, Rozing J, Stijnen T, Hoedemaker PJ, Weening JJ (1988) Mercuric chloride-induced autoimmunity in the Brown Norway rat: cellular kinetics and major histocompatibility complex antigen expression. Am J Pathol 133: 127–138

Batchelor JR, Welsh KI, Mansilla Tinoco R, Dollery CT, Hughes GRV, Bernstein R, Ryan P, Naish PF, Aber GM, Bing RF, Russel GI (1980) Hydralazine-induced systemic lupus erythematosus: influence of HLA-DR and sex on susceptibility. Lancet i: 1107–1109

Beaune PH, Dansette PM, Mansuy D, Kiffel L, Finck M, Amar C, Leroux JP, Homberg JC (1987) Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P-450 that hydroxylates the drug. Proc Natl Acad Sci USA 84: 551–555

Bekesi JG, Holland JF, Anderson HA, Fischbein AS, Rom W, Wolf MS, Selekoff IJ (1978) Lymphocyte function of Michigan dairy farmers exposed to polybrominated biphenyls. Science 199: 1207–1209

Bekesi JG, Roboz JP, Solomon S, Fischbein A, Roboz J, Selikoff IJ (1983) Persistent immune dysfunction in Michigan dairy farm residents exposed to polybrominated biphenyls. In: Hadden JW, Chedid L, Dukor P, Spreafico F, Willoughby D (eds) Advances in immunopharmacology 2. Pergamon Press, New York, pp 33–39

Black CM, Welsh KI, Walker AE, Bernstein RM, Catoggio LJ, McGregor AR, Lloyd Jones JK (1983) Genetic susceptibility to scleroderma-like syndrome induced by vinyl chloride. Lancet i: 53–55

Bruton OC (1952) Agammaglobulinaemia. Pediatrics 9: 722–727

Carlstedt-Duke JMB, Harnemo U-B, Hogberg B, Gustafsson JA (1981) Interaction of the hepatic receptor protein for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin with DNA. Biochim Biophys Acta 672: 131–138

Chang KJ, Hsieh KH, Lee TP, Tang SY, Tung TC (1981) Immunologic evaluation of patients with PCB poisoning: determination of lymphocyte subpopulations. Toxicol Appl Pharmacol 61: 58–63

Chang KJ, Hsieh KH, Lee TP, Tung TC (1982a) Immunologic evaluation of patients with polychlorinated biphenyl poisoning: determination of phagocyte Fc and complement receptors. Environ Res 28: 329–334

Chang KJ, Hsieh KH, Tang SY, Tung TC, Lee TP (1982b) Immunologic evaluation of patients with polychlorinated biphenyl poisoning: evaluation of delayed-type skin hypersensitivity response and its relation to clinical status. J Toxicol Environ Health 9: 217–223

Chapel H, Haeney M (1984) Essentials of clinical immunology. Blackwell Scientific Publications, Oxford

Claas FHJ, van Rood JJ (1985) The interaction of drugs and endogenous substances with HLA class-I antigens. Prog Allergy 36: 135–150

Clark DA, Gauldie J, Szewczuk MR, Sweeney G (1981) Enhanced suppressor cell activity as a mechanism of immunosuppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (41275). Proc Soc Exp Biol Med 168: 290–299

Clark DA, Sweeney G, Safe S, Hancock E, Kilburn DG, Gauldie J (1983) Cellular and genetic basis for suppression of cytotoxic T-cell generation by haloaromatic hydrocarbons. Immunopharmacology 6: 143–153

Coblyn JS, Weinblatt M, Holdsworth D, Glass D (1981) Gold induced thrombocytopenia: a clinical and immunogenetic study of twenty-three patients. Ann Int Med 95: 178–181

Cohen I (1986) Regulation of autoimmune disease: physiological and therapeutic. Immunol Rev 94: 5–21

Dawkins RL, Christiansen FT, Zilko PJ (eds) (1982) In: Immunogenetics in rheumatology. Musculoskeletal diseases and D-penicillamine. Excerpta Medica, Amsterdam, pp 280–375

Dean JH, Luster MI, Boorman GA, Luebke RW, Lauer LD (1980) The effect of adult exposure to diethylstilbestrol in the mouse: alterations in tumor susceptibility and host resistance parameters. J Reticuloendothel Soc 28: 571–584

Dean JH, Thurmond LM, Lauer LD, House RV (1987) Comparative toxicology and correlative immunotoxicology in rodents. In: Burger EJ, Tardiff RG, Bellanti JA, (eds) Environmental chemical exposures and immune system integrity. Princeton Scientific Publishing Co., Princeton, pp 85–100

Descotes J (1988) Immunotoxicology of drugs and chemicals, 2nd edn. Elsevier, Amsterdam

DeSwarte RD (1980) Drug allergy. In: Patterson R (ed) Allergic diseases. JB Lippincott Company, Philadelphia, Toronto, pp 452–583

DeSwarte RD (1986) Drug allergy. An overview. Clin Rev Allergy 4: 143–169

De Weck AL (1983) Penicillins and cephalosporins. In: de Weck AL, Bundgaard JO (eds) Handbook of experimental pharmacology, Vol 63: Allergic reactions to drugs. Springer, Berlin, pp 423–482

DiGeorge AM (1968) Congenital absence of the thymus and its immunologic consequences. Concurrence with congenital hypoparathyroidism. In: Bergsma D, McKusick FA (eds) Immunologic deficiency diseases in man. Williams and Wilkins, Baltimore

Dighiero G, Lymberi J, Marie JC, Rouyse S, Butler-Browne GS, Whalen RG, Avrameas S (1983) Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. J Immunol 135: 2267–2271

Donker AJ, Venuto RC, Vladutiu AO, Brentjens JR, Andres GA (1984) Effects of prolonged administration of D-penicillamine or captropril in various strains of rats. Brown Norway rats treated with D-penicillamine develop autoantibodies, circulating immune complexes, and disseminated intravascular coagulation. Clin Immunol Immunopathol 30: 142–155

Druet P, Sapin C, Druet E, Hirsch F (1983) Genetic control of mercury-induced immune response in the rat. In: Porter GA (ed) Nephrotoxic mechanisms of drugs and environmental toxins. Plenum Medical Book Company, New York, London, pp 425–435

Druet P, Hirsch F, Pelletier L, Druet E, Baran D, Sapin C (1987) Mechanisms of chemical-induced glomerulonephritis. In: Fowler BA (ed) Mechanisms of cell injury: implications for human health. Dahlem Konferenzen. John Wiley and Sons Ltd, Chichester, pp 153–173

Emery P, Panayi S, Huston G, Welsh KI, Mitchell SC, Shah RR, Idel JR, Smith RL, Waring RH (1984) D-Penicillamine induced toxicity in rheumatoid arthritis: the role of sulphoxidation status and HLA-DR3. J Rheumatol 11: 626–632

Faith RE, Luster MI, Kimmel CA (1979) Effect of chronic developmental lead exposure on cell-mediated immune functions. Clin Exp Immunol 35: 413–420

Fillastre JP, Mery JP, Morel-Maroger L, Kanfer A, Godin M (1985) Drug-induced glomerulonephritis. In: Solez K, Whelton A (eds) Acute renal failure. Marcel Dekker, New York, Basel, pp 389–407

Fleuren GJ, DeHeer E, Burgers JV, Osnabrugge C, Hoedemaeker PJ (1985) Mercuric chloride-induced autoimmune glomerulopathy in BALB/c mice. (Abstract). Kidney Int 28: 702

Fraker PJ (1979) The antibody-mediated and delayed-type hypersensitivity response of mice exposed to polybrominated biphenyls. Toxicol Appl Pharmacol 53: 1–7

Fudenberg HH, Stites DP, Caldwell JL, Wells JV (eds) (1980) Basic and clinical immunology. Lange Medical Publications, Los Altos

Gainer JH (1977) Effects of heavy metals and of deficiency of zinc on mortality rates in mice infected with encephalomyocarditis virus. Am J Vet Res 38: 869–874

Gaworski CL, Sharma RP (1978) The effects of heavy metals on [^{3}H]thymidine uptake in lymphocytes. Toxicol Appl Pharmacol 46: 305–313

Gitlin D, Vawter G, Craig JM (1964) Thymic alymphoplasia and congenital aleukocytosis. Pediatrics 33: 184–192

Glanzmann E, Rinicker P (1950) Essentielle Lymphocytophthise. Ein neues Krankheitsbild aus der Säuglingspathologie. Ann Pediat (Basel) 175: 1–32

Gleichmann H, Gleichmann E (1987) Mechanisms of autoimmunity. In: Berlin A, Dean J, Draper MH, Smith EMB, Spreafico F (eds) Immunotoxicology. Martinus Nijhoff Publishers, Dordrecht, pp 39–60

Gleichmann H, Klinkhammer C (1988) Predictive tests in immune reactions to drugs. In: Estabrook RW, Lindenlaub E, Oesch F, de Weck AL (eds) Toxicological and immunological aspects of drug metabolism and environmental chemicals. Symposia Medica Hoechst 22, Schattauer, Stuttgart, New York, pp 535–548

Gleichmann E, Pals ST, Rolink AG, Radaszkiewicz T, Gleichmann H (1984) Graft-versus-host reactions: clues to the etiopathology of a spectrum of immunological diseases. Immunology Today 5: 324–332

Gleichmann E, Vohr H-W, Stringer C, Nuyens J, Gleichmann H (1988) Testing the sensitization of T cells to chemicals. From murine graft-versus-host reactions (GVHRs) to chemical-induced GVH-like immunological diseases. In: Kammüller ME, Bloksma N, Seinen W (eds) Autoimmunity and toxicology. Immunedysregulation induced by drugs and chemicals. Elsevier, Amsterdam, pp 363–390

Greenlee WF, Poland A (1979) Nuclear uptake of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in C57BL/6J and DBA/2J mice. J Biol Chem 254: 9814–9821

Guillaume JC, Roujeau JC, Touraine R (1984) Lupus systèmique après prothèses mammaires. Ann Derm Verner 111: 703–704

Hakala M, Van Assendelft AHW, Ilonen J, Javala S, Tiilikainen A (1986) Association of different HLA antigens with various toxic effects of gold salts in rheumatoid arthritis. Ann Rheum Dis 45: 177–182

Hemphill FE, Kaeberle ME, Buck WB (1971) Lead suppression of mouse resistance to *Salmonella typhimurium*. Science 172: 1031–1032

Hindsill RD, Couch DC, Spiers RS (1980) Immunosuppression in mice induced by dioxin (TCDD) in feed. J Environ Pathol Toxicol 3: 401–425

Hohlfeld R, Kalies I, Kohleisen B, Heininger K, Conti-Tronconi B, Toyka K (1986) Myasthenia gravis: stimulation of antireceptor antibodies by autoreactive T cell lines. Neurology 36: 618–621

Hultman P, Enestrom S (1987) The induction of immune complex deposits in mice by peroral and parenteral administration of mercuric chloride: strain dependent susceptibility. Clin Exp Immunol 67: 283–292

Hurtenbach U, Gleichmann H, Nagata N, Gleichmann E (1987) Immunity to D-penicillamine: genetic, cellular, and chemical requirements for induction of popliteal lymph node enlargement in the mouse. J Immunol 139: 411–416

Kammüller ME, Seinen W (1988) Structural requirements for hydantoins and 2-thiohydantoins to induce lymphoproliferative popliteal lymph node reactions in the mouse. Int J Immunopharmacol 10: 997–1010

Kammüller ME, Thomas C, De Bakker JM, Bloksma N, Seinen W (1989) The popliteal lymph node assay in mice to screen for the immune dysregulating potential of chemicals. A preliminary study. Int J Immunopharmacol 11: 293–300

Kerkvliet NI (1984) Halogenated aromatic hydrocarbons (HAH) as immunotoxicants. In: Kende M, Grainer J, Chirigos M (eds) Chemical regulation of immunity in veterinary medicine. Alan R Liss, New York, pp 369–387

Kerkvliet NI, Baecher-Steppan L (1982) Immunotoxicology studies on lead: Effects of exposure on tumor growth and cell-mediated tumor immunity after syngeneic or allogeneic stimulation. Immunopharmacology 4: 213–224

Kimata H, Shinomiy K, Mikawa H (1983) Selective enhancement of human IgE production in vitro by synergy of pokeweed mitogen and mercuric chloride. Clin Exp Immunol 53: 183–191

Kimber I, Jackson JA, Stonard MD (1986a) Failure of inorganic lead exposure to impair natural killer (NK) cell and T lymphocyte function in rats. Toxicol Lett 31: 211–218

Kimber I, Stonard MD, Gidlow DA, Niewola Z (1986b) Influence of chronic low-level exposure to lead on plasma immunoglobulin concentration and cellular immune function in man. Int Arch Occup Environ Health 57: 117–125

Klinkhammer C, Popowa P, Gleichmann H (1988) Specific immunity to the diabetogen streptozotocin: cellular requirements for induction of lymphoproliferation. Diabetes 37: 74–80

Knutsen AP (1984) Immunologic effects of TCDD exposure in humans. Bull Environ Contam Toxicol 33: 673–681

Koller LD (1973) Immunosuppression induced by lead, cadmium and mercury. Am J Vet Res 34: 1457–1458

Koller LD, Kovacic S (1974) Decreased antibody formation in mice exposed to lead. Nature 250: 148–150

Koller LD, Exon JH, Roan JG (1976) Humoral antibody response in mice after single dose exposure to lead or cadmium. Proc Soc Exp Biol Med 151: 339–342

Kumagai Y, Shiokawa Y, Medsger TA Jr, Rodnan GP (1984) Clinical spectrum of connective tissue disease after cosmetic surgery. Observations of eighteen patients and a review of the Japanese literature. Arthr Rheum 27: 1–12

Lawrence DA (1981) In vivo and in vitro effects of lead on humoral and cell-mediated immunity. Infect Immun 31: 136–143

Lee TP, Chang KJ (1985) Health effects of polychlorinated biphenyls. In: Dean JH, Luster MI, Munson AE, Amos H (eds) Immunotoxicology and immunopharmacology. Raven Press, New York, pp 415–422

Lockwood CM (1987) Association of alkaline phosphatase with an autoantigen: recognition by circulating anti-neutrophil antibodies in systemic vasculitis. Lancet i: 716–719

Londei M, Botazzo GF, Feldmann M (1985) Human T cell clones from autoimmune thyroid glands: specific recognition of autologous thyroid cells. Science 228: 85–89

Loose D, Silkworth JB, Pittman KA, Benitz KF and Mueller W (1978) Impaired host resistance to endotoxin and malaria in polychlorinated biphenyl- and hexachlorobenzene-treated mice. Infect Immun 20: 30–35

Luster MI, Faith RE, Kimmel CA (1978a) Depression of humoral immunity in rats following chronic developmental lead exposure. J Environ Pathol Toxicol 1: 397–402

Luster MI, Faith RE, Moore JA (1978b) Effects of polybrominated biphenyls (PBB) on immune response in rodents. Environ Health Perspect 23: 227–232

Luster MI, Boorman GA, Harris MW, Moore JA (1980) Laboratory studies on polybrominated biphenyl-induced alterations following low-level chronic or pre/postnatal exposure. Int J Immunopharmacol 2: 69–80

Malinow MR, Bardana EJ Jr, Pirofsky B, Craig S, MacLaughlin P (1982) Systemic lupus erythematosus-like syndrome in monkeys fed alfalfa sprouts: role of a nonprotein amino acid. Science 216: 415–417

Merk H, Schneider R, Scholl P (1988) Lymphocyte stimulation by drug-modified microsomes. In: Estabrook RW, Lindenlaub E, Oesch F, de Weck AL (eds) Toxicological and immunological aspects of drug metabolism and environmental chemicals. Symposia Medica Hoechst 22, Schattauer, Stuttgart New York, pp 211–219

Miller K, Scott MP, Foster JR (1984) Thymic involution in rats given diets containing dioctyltin dichloride. Clin Immunol Immunopathol 30: 62–70

Mirtschewa J, Nürnberger W, Hallmann B, Stiller-Winkler R, Gleichmann E (1987) Genetically determined susceptibility of mice to $HgCl_2$-induced antinuclear antibodies (ANA) and glomerulonephritis. (Abstract). Immunobiology 175: 323–324

Nagata N, Hurtenbach U, Gleichmann E (1986) Specific sensitization of Lyt-1$^+$2$^-$ T cells to spleen cells modified by the drug D-penicillamine or a stereoisomer. J Immunol 136: 136–142

Nebert DW, Goujon FM, Gielen JE (1972) Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. Nature New Biol 236: 107–110

Neilan BA, O'Neill K, Handwerger BS (1983) Effect of low-level lead exposure on antibody-dependent and natural killer cell-mediated cytotoxicity. Toxicol Appl Pharmacol 69: 272–275

Okey AB, Bondy GP, Mason ME, Kahl GF, Eisen HJ, Guenthner TM, Nebert DW (1979) Regulatory gene product of the Ah locus. Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. J Biol Chem 254: 11636–11648

Okey AB, Bondy GP, Mason ME, Nebert DW, Forster-Gibson CJ, Muncan J, Dufresne MJ (1980) Temperature-dependent cytosol-to-nucleus translocation of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in continuous cell culture lines. J Biol Chem 255: 11415–11422

Panayi GS, Huston G, Shah RR, Mitchell SC, Idle Smith JR, Waring RH (1983) Deficient sulphoxidation status and D-penicillamine toxicity. Lancet i: 414

Parker CW (1980) Drug allergy. In: Parker CW (ed) Clinical immunology. WB Saunders Co., Philadelphia, pp 2219–2260

Penn I (1977) Development of cancer as a complication of clinical transplantation. Transplant Proc 9: 1121–1127

Penn I (1985) Neoplastic consequences of immunosuppression. In: Dean JH, Luster MI, Munson AE, Amos H (eds) Immunotoxicology and immunopharmacology. Raven Press, New York, pp 79–89

Penninks AH, Seinen W (1985) On the mechanism of dialkyltin-induced thymus involution. Immunopharmacology 10: 1–10

Penninks AH, Seinen W (1987) Immunotoxicity of organotin compounds. A cell biological approach to dialkyltin induced thymus atrophy. In: Berlin A, Dean JH, Draper MH, Smith EMB, Spreafico F (eds) Immunotoxicology. Martinus Nijhoff Publishers, Dordrecht, pp 258–278

Pereyo N (1980) Tartrazine-induced lupus. Shock Lett 30: 1

Perrier P, Raffoux C, Thomas Ph, Tomisier JN, Busson M, Gaucher A, Streiff F (1985) HLA antigens and toxic reactions to sodium aurothiopropanol sulphonate and D-penicillamine in patients with rheumatoid arthritis. Ann Rheum Dis 44: 621–624

Pietsch P, Allmeroth M, Gleichmann E, Vohr HW (1987) Increased synthesis of IgE, but not IgM, in strains of mice susceptible to $HgCl_2$. (Abstract). Immunobiology 175: 324

Polak L, Barnes JM, Turk JL (1968) The genetic control of contact sensitization to inorganic metal compounds in guinea-pigs. Immunology 14: 707–711

Poland A, Glover E, Kende AS (1976) Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. J Biol Chem 251: 4936–4946

Ptak W, Rewicka M, Marcinkiewicz J (1984) Induction of "allogeneic effect"-like reaction by syngeneic TNP-modified lymphoid cells. Immunobiology 166: 368–381

Pujol-Borell R, Todd I, Doshi M, Botazzo GF, Sutton R, Gray D, Adolf GR, Feldmann M (1987) HLA class II induction in human islet cells by interferon-gamma plus tumour necrosis factor or lymphotoxin. Nature 326: 304–306

Reggiani G (1978) Medical problems raised by TCDD contamination in Seveso, Italy. Arch Toxicol 4: 161–188

Reidenberg MM, Durant PJ, Harris RA, Debocardo G, Lahita R, Stenzel KH (1983) Lupus erythematosus-like disease due to hydrazine. Am J Med 75: 365–370

Robinson GCJ, Balazs T, Egorov IK (1986) Mercuric chloride-, gold sodium thiomalate-, and D-penicillamine-induced antinuclear antibodies in mice. Toxicol Appl Pharmacol 86: 159–169

Seinen W, Willems MI (1976) Toxicity of organotin compounds. I. Atrophy of thymus and thymus-dependent lymphoid tissue in rats fed di-*n*-octyltindichloride. Toxicol Appl Pharmacol 35: 63–75

Seinen W, Vos JG, Van Spanje I, Snoek M, Brands R, Hooykaas H (1977a) Toxicity of organotin compounds II. Comparative in vivo and in vitro studies with various organotin and organolead compounds in different animal species with special emphasis on lymphocyte cytotoxicity. Toxicol Appl Pharmacol 42: 197–212

Seinen W, Vos JG, Van Krieken P, Penninks A, Brands R, Hooykaas H (1977b) Toxicity of organotin compounds. III. Suppression of thymus-dependent immunity in rats by di-*n*-butyltin dichloride and di-*n*-octyltin dichloride. Toxicol Appl Pharmacol 42: 213–224

Seinen W, Vos JG, Brands R, Hooykaas H (1979) Lymphocytotoxicity and immunosuppression by organotin compounds. Suppression of GVH activity, blast transformation and E-rosette formation by di-*n*-butyltin dichloride and di-*n*-octyltin dichloride. Immunopharmacology 1: 343–355

Sell S (1987) Immunology, immunopathology, and immunity, 4th edition. Elsevier, New York, pp 310 and 320

Sharma RP, Kociba RJ, Gehring PJ (1978) Immunotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals. Toxicol Appl Pharmacol 45: 333 (A.266)

Shigematsu N, Ishimarus S, Saito R, Ikeda T, Matsuba K, Sugiyama K, Masuda Y (1978) Respiratory involvement in polychlorinated biphenyls poisoning. Environ Res 16: 92–100

Silkworth JB, Vecchi A (1985) Role of the Ah receptor in halogenated aromatic hydrocarbon immunotoxicity. In: Dean JH, Luster MI, Munson AE, Amos H (eds) Immunotoxicology and immunopharmacology. Raven Press, New York, pp 263–275

Stark M, Zaidi S, Hilscher B, Hilscher W, Gleichmann E (1988) High- and low-responder mouse strains with respect to silica-induced fibrosis (Abstract). Immunobiology 178: 100–101

Stejskal VDM, Olin R, Forsbeck M (1986) The lymphocyte transformation test for diagnosis of drug-induced occupational allergy. J Allergy Clin Immunol 77: 411–426

Stiller-Winkler R, Radaszkiewicz T, Gleichmann E (1988) Immunopathological signs in mice treated with mercury compounds. I. Popliteal lymph node reactions to mercury compounds: responder and non-responder mouse strains to $HgCl_2$. Int J Immunopharmacol 10: 475–484

Taugner M, Schütz R (1966) Beitrag zur Quecksilber-Allergie. Dermatologica 133: 245–261

Thomas PJ, Hindsill RD (1978) Effect of chlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicol Appl Pharmacol 44: 41–52

Tubbs RR, Gephardt GN, McMahon JT, Pohl MC, Vidt DG, Barenberg SA, Valenzuela R (1982) Membranous glomerulonephritis associated with industrial mercury exposure. Study of pathogenetic mechanisms. Am J Clin Pathol 77: 409–413

Victorino RMM, Maria VA (1985) Modifications of the lymphocyte transformation test in a case of drug-induced cholestatic hepatitis. Diagnostic Immunol 3: 177–181

Vogeler S, Gleichmann E (1988) Oral treatment of mice with the drug D-Penicillamine (D-Pen) haptenates their peritoneal macrophage and primes their T cells (Abstract). Immunobiology 178: 71

Vos JG, Moore JA (1974) Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Int Arch Allergy 47: 777–794

Vos JG, Van Driel-Grootenhuis L (1972) PCB-induced suppression of the humoral and cell-mediated immunity in guinea pigs. Sci Total Environ 1: 289–295

Vos JG, Moore JA, Zinkl JG (1973) Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. Environ Health Perspect 5: 149–162

Vos JG, Kreeftenberg JG, Engel HWB, Minderhoud A, Van Noorlejansen LM (1978) Studies on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced immune suppression and decreased resistance to infection: endotoxin sensitivity, serum zinc concentrations and effects of thymosin treatment. Toxicology 9: 75–86

Vos JG, De Klerk A, Krajnc EI, Kruizinga W, Van Ommen B, Rozing J (1984) Toxicity of *bis*(tri-*n*-butyltin)oxide in the rat. II. Suppression of thymus-dependent immune response and of parameters of non-specific resistance after short-term exposure. Toxicol Appl Pharmacol 75: 387–408

Webster ADB (1983) Immunodeficiency disease. In: Holborow EJ, Reeves WG (eds) Immunology in medicine, 2nd Edn. Grune and Stratton, London pp 223–241

Weening JJ, Hoedemaeker J, Bakker WW (1981) Immunoregulation and antinuclear antibodies in mercury-induced glomerulopathy in the rat. Clin Exp Immunol 45: 64–71

Weigle WO (1980) Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv Immunol 30: 159–275

Wooley PH, Griffin J, Panayi GS, Batchelor JR, Welsh KI, Gibson TJ (1980) HLA-DR antigens and toxic reaction to sodium aurothiomalate and D-penicillamine in patients with rheumatoid arthritis. New Engl J Med 303: 300–302

Zaidi SH, Hilscher B, Hilscher W, Brassel D, Grover R (1979) Vergleichende morphometrische und autoradiographische Untersuchungen quarzinduzierter Läsionen bei nu/nu-Mäusen und Kontrollmäusen. In: Ergebn Unters Geb Staub- und Silikosebekämpfung im Steinkohlenbergbau. Silikosebericht Nordrhein-Westfalen, Vol. 12, Verlag Glückauf, Essen, pp 187–192

Ziegler V, Haustein U-F, Mehlhorn J, Munzberger H, Rennau H (1986) Quarzinduzierte Sklerodermie. Sklerodermie-ähnliches Syndrom oder echte progressive Sklerodermie? Dermatol Monatsschr 172: 86–90

Received February 20, 1989/Accepted February 28, 1989

Screening and function studies in immunotoxicity testing

J. G. Vos[1]

SUMMARY

After a short introduction of various chemicals of environmental concern that have been shown to alter cell-mediated or humoral immune responses, a screening procedure is given to detect possible immunotoxic properties of chemicals. The different parameters in this screening programme include growth, weight and histology of lymphoid and endocrine organs, haematology, as well as serum immunoglobulin concentrations.

Next, different functional tests are discussed to assess the cell-mediated immunity, the humoral immunity, and the phagocytosis by macrophages in the rat. These tests should be performed when in a screening study an indication of immunotoxicity is found at a relevant dose level. The aim of functional assessment is to determine the functional significance of an effect on the immune response. As the data available at present clearly show that the developing organism is more at risk to the immunomodulating effects of different chemicals than the corresponding adult, functional assessment of immune effects should preferably be carried out after combined pre- and postnatal exposure.

INTRODUCTION

Soon after the identification of polychlorinated biphenyls (PCB) in tissues of fish and wildlife in the Netherlands (9), studies were initiated at the Institute of Veterinary Pharmacology and Toxicology to determine the toxicity of commercial PCB preparations. Following the observation of atrophy of lymphoid tissues in the spleen of chickens (24), and in the thymus (the central lymphoid organ of the cell-mediated immunity), spleen and lymph nodes of rabbits (21), the significance of the effect of PCB on the immune system was functionally assessed in the guinea pig. These studies (22) have clearly shown that PCB exposure suppresses the thymus-dependent humoral immunity (depressed serum antibody titers to tetanus toxoid and reduction of the number of tetanus antitoxin producing cells in lymph nodes) as well as the cell-mediated immunity (delayed-type hypersensitivity to tuberculin).

Prompted by the effects observed in PCB exposed animals, the related polybrominated biphenyls (PBB) were studied. In chickens, dietary exposure to PBB induced lymphoid depletion in the bursa of Fabricius (the central lymphoid organ of the humoral immunity) and spleen. Upon functional assessment, depressed prim-

[1] Laboratory of Pathology, National Institute of Public Health, P.O. Box 1, 3720 BA, Bilthoven, the Netherlands.

ary and secondary immune responses to tetanus toxoid were recorded in PBB exposed guinea pigs (23). In addition, lymphoid depletion was observed in the thymic cortex and in the splenic follicles and periarteriolar lymphocyte sheaths. There was increased interest in PBB with regards to its effect on the immune system after an accident occurred in 1973 in the state of Michigan, when a fire retardant consisting primarily of PBB was inadvertently substituted for magnesium oxide food supplement for livestock (4). Clinical observations of exposed cattle suggested that infections were often present.

With regard to immune effects, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) has been the most thoroughly studied halogenated aromatic hydrocarbon. TCDD is a highly toxic impurity that may be formed during the production of 2, 4, 5-trichlorophenol. Severe atrophy of the thymus has been reported as occurring in almost all laboratory animals exposed to sublethal doses of TCDD. Following the first report of TCDD induced thymic atrophy in the rat, depression of various cell-mediated immune parameters as well as thymus-dependent humoral immune responses was demonstrated in guinea pigs, mice, and rats (25, 31). Significant depression in the two latter species occurred only after exposure during the pre- and postnatal period.

Various organometallic compounds have been studied in respect of their potential immunotoxic properties. Of these different chemicals, dialkyltin compounds have been the most intensively studied, following the initial observation that di-n-octyl tin dichloride (DOTC) caused a severe thymic atrophy in the rat as result of a selective lymphocytotoxicity (14). Upon functional assessment, different parameters of both the cell-mediated and the thymus-dependent humoral immunity were suppressed by DOTC and the related compound di-n-butyl tin dichloride (DBTC). The immune suppression was most pronounced in rats that were exposed immediately after birth (15-17). Based on the results obtained with the different compounds listed above, the following methods are now used in the National Institute of Public Health to screen chemicals for possible immune effects, and to assess functionally the immune system of rats which are exposed to toxic chemicals or drugs.

Detailed information on immune effects of PCB, PBB, TCDD, dialkyl tins, and other compounds is given elsewhere (7, 12, 13, 20, 23, 28).

SCREENING TESTS TO DETECT IMMUNOTOXICITY

It is well established that the most profound effects of compounds which interfere with the immune response occur when the animal is confronted with the compound during the ontogenesis of the lymphoid system. A sensitive system to detect effects on the immune system is, therefore, a reproduction study which includes a thorough evaluation of the lymphoid system. However, this does not imply that studies to detect such effects should always be conducted in animals during the developmental phase of the immune system. For practical reasons, initial assessment could be done in a 3-week range-finding study or in a 3-month semichronic toxicity study.

During these experiments body weight gain and food intake are recorded. At the termination of the toxicity study, thymus, spleen, lymph nodes (popliteal and mesenteric nodes) are examined macroscopically, weighed, and processed for histopathological examination. For the determination of peripheral lymphocyte and monocyte numbers (as precursors of macrophages) total and differential leucocyte counts are carried out. The concentrations of the main serum immunoglobulin classes (e.g. IgM and IgG) can be measured by the enzyme-linked immunosorbent assay (ELISA) (27).

From these different parameters (weight gain, food intake, weight and histology of lymphoid organs, peripheral blood counts, serum IgM and IgG levels) a conclusion may be reached on whether the chemical has an effect on the immune system. Such an effect can be direct or indirect (secondary to an effect elsewhere, e.g. caused by malnutrition or an

altered endocrine balance). Especially an interaction of the chemical with the endocrine system which can indirectly cause an effect on the immune system should be considered, as various hormones (in particular glucocorticosteroids) do modify the immunological responses (20, 33). For this reason, pituitary gland, thyroid, adrenals, testes, and ovaries are also weighed an examined microscopically. If the effect on the immune system cannot be attributed to an indirect effect of which the functional significance is known, and the effect is a sensitive parameter, functional studies should be carried out.

Using the approach here described, a total of 17 different pesticides were recently screened in 3-week toxicity studies (M. J. van Logten and J. G. Vos, unpublished data). Of these compounds, three (triphenyl tin hydroxide, lead arsenate, and captan) had a significant effect on one of the various immune parameters as the most sensitive criterium. With these three chemicals, immune function tests are in progress in rats exposed after weaning as well as in rats exposed pre- and postnatally.

FUNCTION TESTS OF THE IMMUNE SYSTEM

Function studies of the immune system are necessary to gain an insight into the functional significance of the chemically induced effect on the immune system found in a routine study, in order to evaluate the potential risk of the chemical. Subtle effects on immune responses will be more easily detected if the animal is confronted with the chemical during the developmental phase of the lymphoid organs. If the chemical passes the placenta and is excreted in the milk, e.g. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (25) and hexachlorobenzene (30), pre- and postnatal maternal treatment is probably the most sensitive test system to detect an altered immune response. For chemicals which do not readily pass the placenta and are not readily excreted in the milk, e.g. di-n-butyl tin dichloride (16), pups can be treated postnatally by oral intubation. Function studies of the pups could be performed at the time of weaning or later.

The choice of function tests depends mainly on the effects seen in the routine toxicity study: if thymic atrophy is the main characteristic, the cell-mediated response should be studied first. In cases where the effect is primarily on serum immunoglobulins, one should test the capacity of the animal to generate a humoral immune response. Before starting function tests that allow of a separate study of the different phases of the immune response, one should obtain an insight into whether the overall response is impaired. For this purpose it is particularly useful to determine whether the resistance to infection is impaired by the chemical. *In vivo* and *in vitro* function tests will be successively described for the cell-mediated immunity, the humoral immune response to T-cell-dependent and T-cell-independent antigens, and the phagocytizing capacity of macrophages. Comprehensive information on these and other techniques is given elsewhere (1, 11, 32).

A. Cell-mediated Immunity

1. *Resistance to Listeria monocytogenes infection*

The resistance to *Listeria monocytogenes* is a combination of non-specific phagocytosis which inhibits or kills the growth of the organism during the first two days after infection, and cell-mediated immunity which starts operating from day 2 post-infection (3, 19). All phases of the cell-mediated immune response are involved in the acquired resistance to *Listeria*, including the participation of activated macrophages in the killing of *Listeria* in the effector phase. Animals are intravenously injected with *Listeria monocytogenes*. Criteria used to assess the resistance to this type of infection are mortality rates in control animals compared with those in the animals treated with the chemical, or bacterial enumeration in the spleen at days, 4, 6, or later after inoculation, when the cell mediated immunity interrupts the growth of the organism *in vivo*. Spleens of the injected animals are homogenized, and serial di-

lutions of each homogenate are plated to determine the viable counts of *Listeria* (19). Regarding the use of mortality rates as a criterium some doubts remain, since recent data indicate that athymic nude rats (unpublished data) or mice (6) are more resistant to *L. monocytogenes* infection than their thymus-bearing litter mates. Bacterial enumeration in spleen after a sublethal dose of *Listeria* therefore seems the best parameter by which to assess the acquired resistance to *Listeria*.

2. *Rejection of allografts*

In this technique, skin of inbred rats is transplanted to recipients that differ from each other at the major histocompatibility (Ag-B) locus or other important histocomptability loci (5). Subtle suppressive effects on cellular immunity may perhaps be missed, since considerable suppression is required to prolong the allograft rejection time by one or two days. A rapid method of grafting skin on tails is described by Bailey and Usama (2).

3. *Delayed-type hypersensitivity*

An important but not very sensitive test from measuring cell-mediated immunity is the delayed cutaneous hypersensitivity reaction to tuberculin (8). In this test, animals are preferably sensitized with a subcutaneous injection, in the foot pad, of an oil suspension containing killed *Mycobacterium tuberculosis* (H37Ra adjuvant), intradermally challenged with tuberculin PPD during the second and third week after sensitization, and measuring, with calipers, the diameter and thickness of the skin reaction after 24 and 48 h. as. parameter of cell-mediated immunity. An alternative and probably more objective and sensitive method for measuring tuberculin skin hypersensitivity in rats (10) is based on the radioactive labelling, with tritium thymidine, of bone marrow precursors of monocytes, which cells accumulate in delayed-type hypersensitivity reactions and are measured by liquid scintillation counting of a biopsy specimen of the challenged ear. A disadvantage of this test is the *in vivo* use of a radioactive label. As an alternative, the thickness of the delayed reaction in the ear can be measured with calipers. Instead of assaying the hypersensitivity to tuberculin, similar delayed reactions can be elicited by the using of a protein antigen (ovalbumin). Rats are sensitized in the foot pads with a mixture of ovalbumin in H37Ra adjuvant, and intradermal skin test are performed with ovalbumin. The advantage of using ovalbumin is that this protein also induces high antibody titers (cf. Section B1), whereas the optimum immunization dose of ovalbumin for measuring delayed-type hypersensitivity as well as the humoral immune response is the same (26). Thus, parameters of both the cell-mediated and the humoral immunity can be assayed simultaneously in the same animal.

4. *Transformation of lymphocytes by PHA and Con A*

The ability of lymphocytes to transform and incorporate labelled thymidine in DNA following nonspecific *in vitro* stimulation with the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) is a measure of T-cell function, as shown by the absence of a response of spleen cells from athymic nude rats (29), and permits of separate analysis of the adaptive phase of the immune response. In contrast to activation by specific antigens, these mitogens activate a relatively high percentage of lymphocytes, probably representing a polyclonal response. A practical and labour-saving microplate culture system is described by Thorpe and Knight (18). These authors also provide data for optimizing culture conditions and labelling technique.

B. Humoral Immunity

1. *Thymus-dependent antibody synthesis to tetanus toxoid or ovalbumin*

The antibody response to tetanus toxoid and ovalbumin is thymus-dependent in the rat (i.e. it needs the cooperation of so-called T-helper cells), as athymic nude rats do not generate an IgM or IgG antibody response to these antigens (29).

Rats are immunized with tetanus toxoid intravenously or in the footpad with a mixture of H37Ra adjuvant and ovalbumin.

As discussed in Section A3 the advantage of using ovalbumin as antigen is that both the humoral and the cell-mediated immunity can be assayed in the same animal. In addition, ovalbumin is highly immunogenic in the rat and generates, besides IgM and IgG, also antibodies of the IgE class. Serum antibody titers are determined by the enzyme-linked immunosorbent assay (ELISA) as described elsewhere for tetanus toxoid (27) and ovalbumin (26).

2. *Thymus-independent antibody synthesis to LPS*

The antibody response to *Escherichia coli* lipopolysaccharide (LPS) does not require the cooperation by T-helper cells, since a similar antibody response is achieved in the athymic nude rat as compared with thymus-bearing litter mates (29). Additional evidence that the humoral immune response to LPS *E. coli* is a thymus-independent phenomenon comes from the observation that immunological memory did not develop after the primary immunization with LPS. LPS is poorly immunogenic in the rat, but by means of ELISA it is possible to determine reasonable serum titers (27).

3. *Transformation of lymphocytes by LPS*

E. coli lipopolysaccharide (LPS) is a B-cell mitogen (T-cell independent) in the rat, as a normal response is seen in spleen cells from athymic nude rats (29). Information on LPS stimulation of rat lymphocytes is scarce, which may be due to the fact that the response of cell suspensions of lymphoid organs of the rat to this mitogen is poor (34).

C. Phagocytosis by Macrophages

Clearance of Listeria monocytogenes

As discussed in section A1, the resistance to *Listeria monocytogenes* is a combination of nonspecific phagocytosis and cell-mediated immunity. Nonspecific phagocytosis and killing can be measured shortly (day 1 and 2) after the intravenous inoculation of *Listeria* organisms, at a time when the cell-mediated immunity is not yet developed. Spleens of the injected animals are homogenized, and serial dilutions of each homogenate are plated to determine the viable counts of *Listeria*, which is a measure of the phagocytic and killing activity of macrophages.

REFERENCES

1. Bach, J. F.: The mode of action of immunosuppressive agents. North-Holland Publishing Company, Amsterdam, 1975.
2. Bailey, D. W. en Usama, B.: A rapid method of grafting skin on tails of mice. *Transplant. Bull.*, 7, *424, (1960).*
3. Cheers, C., McKenzie, I. F. C., Pavlov, H., Waid, C., and York, J.: Resistance and susceptibility of mice to bacterial infection: course of listeriosis in resistant or susceptible mice. *Infect. Immun.* 19, *763 (1978).*
4. Dunkel, A. E.: An updating on the polybrominated biphenyl disaster in Michigan. *J. Am. Vet. Med. Ass.*, 167, *838, (1975).*
5. Elkins, W. L. and Palm, J.: Identification of a single strong histocompatibility locus in the rat by normal spleen-cell transfer. *An. N. Y. Acad. Sci.*, 129, *573, (1966).*
6. Emmerling, P., Finger, H., and Hof, H.: Cell-mediated resistance to infection with *Listeria monocytogenes* in nude mice. *Infect. Immun.*, 15, *382, (1977).*
7. Faith, R. E., Luster, M. I., and Vos, J. G.: Effects on immunocompetence by chemicals of environmental concern. In: Reviews in Biochemical Toxicology 2 (E. Hodgson, J. R. Bend, and R. M. Philpot, Eds.), Elsevier/North Holland, New York, 173-211, 1980.
8. Flax, M. H. and Waksman, B. H.: Delayed cutaneous reactions in the rat. *J. Immunol.*, 89, *496, (1962).*
9. Koeman, J. H., ten Noever de Brauw, M. C., and de Vos, R. H.: Chlorinated biphenyls in fish, mussels, and birds from the river Rhine and the Netherlands coastal area. *Nature*, 221, *1126, (1969).*
10. Lefford, M. J.: The measurement of tuberculin hypersensitivity in rats. *Int. Arch., Allergy.* 47, *570, (1974).*
11. Mitchell, M. S.: Evaluation of immunosuppressive agents. In: Handbook of Experimental Pharmacology (A. C. Sartorelli, D. G. Johns, Eds.), vol. 38/1, 555-576, Springer Verlag, Berlin, 1974.

deter-

12. Seinen, W.: Immunotoxicity of alkyl tin compounds. Ph.D. thesis, University of Utrecht, 1978.
13. Seinen, W. and Penninks, A. H.: Immune suppression as a consequence of a selective cytotoxic activity of certain organometallic compounds on thymus dependent lymphocytes. *Ann. N. Y. Acad. Sci.*, 320, *499, (1979).*
14. Seinen, W. and Willems, M. I.: Toxicity of organotin compounds. I. Atrophy of thymus and thymus-dependent lymphoid tissue in rats fed di-n-octyl tin dichloride. *Toxicol. Appl. Pharmacol.*, 35, *63, (1978).*
15. Seinen, W., Vos, J. G., Brands, R., and Hooykaas, H.: Lymphocytotoxicity and immunosuppression by organotin compounds. Suppression of GVH reactivity, blast transformation, and E-rosette formation by di-n-butyl tin dichloride and di-n-octyl tin dichloride. *Immunopharmacol.*, 1, *343, (1979).*
16. Seinen, W., Vos, J. G., van Krieken, R., Penninks, A. H., Brands, R., and Hooykaas, H.: Toxicity of organotin compounds. III. Suppression of thymus-dependent immunity in rats by di-n-butyl tin dichloride and di-n-octyl tin dichloride. *Toxicol. Appl. Pharmacol.*, 42, *213. (1977).*
17. Seinen, W., Vos, J. G., van Spanje, I., Snoek, M., Brands, R., and Hooykaas, H.: Toxicity of organotin compounds. II. Comparative *in vivo* and *in vitro* studies with various organotin and organolead compounds in different animal species with special emphasis on lymphocyte cytotoxicity. *Toxicol. Appl. Pharmacol.*, 42, *197. (1977).*
18. Thorpe, P. E. and Knight, S. C.: Microplate culture of mouse lymph node cells. I. Quantification of responses to allogeneic lymphocytes, endotoxins, and phytomitogens. *J. Immunol. Methods.* 5, *387. (1974).*
19. Tripathy, S. P. and Mackaness, G. B.: The effect of cytotoxic agents on the primary immune response to *Listeria monocytogenes. J. Exp. Med.*, 130, 1, (1969).
20. Vos, J. G.: Immune suppression as related to toxicology. *CRC, Crit. Rev. Toxicol.*, 5, *67. (1977).*
21. Vos, J. G. and Beems, R. B.: Dermal toxicity studies of technical polychlorinated biphenyls and fractions thereof in rabbits. *Toxicol. Appl. Pharmacol.*, 19, *617. (1971).*
22. Vos, J. G. and van Driel Grootenhuis, L.: PCB-induced suppression of the humoral and cell-mediated immunity in guinea pigs. *Sci. Total Environ.* 1, *289. (1972).*
23. Vos, J. G. and van Genderen, H.: Toxicological aspects of immunosuppression. In.: Pesticides and the Environment: A Continuing Controversy. (W. B. Deichmann, Ed.). Intercontinental Medical Book, Corp., New York, 527-545, 1973.
24. Vos. J. G. and Koeman, J. H.: Comparative toxicologic study with polychlorinated biphenyls in chickens, with special reference to porphyria, oedema formation, liver necrosis, and tissue residues. *Toxicol. Appl. Pharmacol.*, 17, *656. (1970).*
25. Vos, J. G. and Moore, J. A.: Suppression of cellular immunity in rats and mice by maternal treatment with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. Int. Arch. Allergy, 47, *777. (1974).*
26. Vos, J. G., Boerkamp, J., Buys, J., and Steerenberg, P. A.: Ovalbumin immunity in the rat: simultaneous testing of IgM, IgG, and IgE response measured by ELISA and delayed-type hypersensitivity. *Scand. J. Immunol.*, 12, *289, (1980).*
27. Vos, J. G., Buys, J., Beekhof, P., and Hagenaars, A. M.: Quantification of total IgM and IgG and specific IgM and IgG to a thymus-independent (LPS) and a thymus-dependent (tetanus toxoid) antigen in the rat by enzyme-linked immunosorbent assay (ELISA). *Ann. N. Y. Acad. Sci.*, 320, *518, (1979).*
28. Vos, J. G., Faith, R. E., and Luster, M. I.: Immune alterations. In: Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products (R. D. Kimbrough, Ed.). Elsevier/North Holland, Amsterdam, in press 1980.
29. Vos, J. G., Kreeftenberg, J. G., Kruijt, B. C., Kruizinga, W., and Steerenberg, P.: The athymic nude rat. II. Immunological characteristics. *Clin. Immunol. Immunopathol.*, 15, *229. (1980).*
30. Vos, J. G., van Logten, M. J., Kreeftenberg, J. G., Steerenberg, P. A., and Kruizinga, W.: Effect of hexachlorobenzene on the immune system of rats following combined pre- and postnatal exposure. *Drug Chem. Toxicol.*, 2, *61. (1979).*
31. Vos, J. G., Moore, J. A., and Zinkl, J. G.: Effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on the immune system of laboratory animals. *Environm. Health Perspect.* 5, *149, (1973).*
32. Weir, D. M.: Handbook of Experimental Immunology. 2nd ed., Blackwell Scientific, Oxford, 1973.
33. White, A. and Goldstein, A. L.: Hormonal regulation of host immunity. In: Immunogenicity, Frontiers of Biology (F. Borek, Ed.) vol. 25, 334-364. North-Holland Publishing Company, Amsterdam, 1972.
34. Yoshinaga, M., Yoshinaga, A., and Waksman, B. H.: Regulation of lymphocyte responses *in vitro*. I. Regulatory effect of macrophages and thymus-dependent (T) cells on the response of thymus-independent (B) lymphocytes to endotoxin. *J. Exp. Med.*, 136, *956. (1972).*

E2

REPORTS

Enhanced Cancer Growth in Mice Administered Daily Human-Equivalent Doses of Some H_1-Antihistamines: Predictive In Vitro Correlates

*Lorne J. Brandes, Robert C. Warrington, Randi J. Arron, R. Patricia Bogdanovic, Wei Fang, Gary M. Queen, Douglas A. Stein, Jiangang Tong, Cheryl L. F. Zaborniak, Frank S. LaBella**

Background: Present studies of drug-induced tumor growth promotion have evolved from earlier investigations into the mechanism of action of *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine · HCl, a tamoxifen derivative which potently inhibits lymphocyte mitogenesis in vitro and stimulates tumor growth in vivo. It is thought that potency to bind to intracellular histamine receptors (H_{1C}), some of which are on cytochromes P450, may correlate with tumor growth-promoting activity. ***Purpose:*** We assessed the effectiveness of five in vitro assays in predicting in vivo tumor growth stimulation by the H_1-antihistamines loratadine, astemizole, cetirizine, hydroxyzine, and doxylamine. ***Methods:*** Potency of each agent was ranked 1-5 in each of the following in vitro assays: 1) inhibition of [^{3}H]histamine binding to microsomal H_{1C}, 2) inhibition of histamine binding to microsomal P450, 3) inhibition of the P450-catalyzed demethylation of aminopyrine, 4) inhibition of lymphocyte mitogenesis, and 5) stimulation of tumor colony formation. An overall rank score was assigned to each drug and correlated with tumor growth stimulation in vivo. Two laboratories conducted in vivo studies in a blinded fashion. Female C57BL and C3H mice were given a subcutaneous injection on day 1 of syngeneic B16F10 melanoma cells (5×10^5) or C-3 fibrosarcoma cells (1×10^5), respectively. Mice were randomly assigned to treatment groups, then received a single, daily intraperitoneal injection of an estimated human-equivalent dose (or range of doses) of antihistamine or vehicle control for 18-21 days before being killed. Tumors were surgically removed and wet weights compared statistically among groups. ***Results:*** The cumulative potency of each drug in affecting tumor growth or growth mechanisms in the five in vitro assays ranked as follows: Loratidine and astemizole ranked highest and were equally potent, followed in decreasing order by hydroxyzine, doxylamine, and cetirizine. A significant correlation (r = .97; P<.02) was observed between the rank order of potency of the antihistamines in all five in vitro assays and the rank order to enhance tumor growth in vivo: Loratidine and astemizole significantly (P<.001) promoted the growth of both melanoma and fibrosarcoma, hydroxyzine significantly (P<.001) promoted the growth of melanoma, while doxylamine and cetirizine did not promote the growth of either tumor. ***Conclusion:*** Data demonstrate that the in vitro assays predicted the propensity of each H_1-antihistamine to stimulate cancer growth in vivo. ***Implication:*** These in vitro tests may prove valuable to screen potential tumor growth promoters. [J Natl Cancer Inst 86:770-775, 1994]

We (*1*) reported that, at human-equivalent doses, two nongenotoxic antidepressant drugs, amitriptyline (Elavil, Stuart Pharmaceuticals, Wilmington, Del.) and fluoxetine (Prozac, Dista Division, Eli Lilly and Co., Indianapolis, Ind.), accelerate the growth of cancer in rodents. Subsequently, others (*2,3*) confirmed a similar property for clomipramine (Anafranil, Basel Pharmaceuticals, Summit, N.J.) and desipramine (Norpramin, Marion Merrell Dow Inc., Kansas City, Mo.), both tricyclic analogues of amitriptyline. Our studies of drug-induced tumor growth promotion arose from earlier investigations into the mechanism of action of *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine · HCl (DPPE), a tamoxifen derivative that potently inhibits lymphocyte mitogenesis in vitro (*4*) and stimulates tumor growth in vivo (*5*). Through binding to microsomal and nuclear anti–estrogen-binding sites (AEBS) (*6*), DPPE inhibits the binding of [^{3}H]histamine to AEBS-associated intracellular histamine receptors (H_{1C}) implicated in growth regulation (*7*); some of the microsomal H_{1C} sites are associated with cytochromes P450 (*8*). Tamoxifen, amitriptyline, and fluoxetine also bind potently to H_{1C} (*1,6*); like DPPE, they inhibit mitogenesis in vitro and significantly enhance the growth of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in vivo (*1,9*); we (*5*) have postulated that potency to bind H_{1C} may correlate with tumor growth-promoting activity.

A number of other drugs (*5*), including H_1-antihistamines commonly prescribed

**Affiliations of authors:* L. J. Brandes, Departments of Medicine and Pharmacology and Therapeutics, Faculty of Medicine, Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Canada.

R. C. Warrington, W. Fang, Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada.

R. J. Arron, R. P. Bogdanovic, J. Tong, C. L. F. Zaborniak, Department of Medicine, Manitoba Institute of Cell Biology.

G. M. Queen, D. A. Stein, F. S. LaBella, Department of Pharmacology and Therapeutics, University of Manitoba.

Correspondence to: Lorne J. Brandes, M.D., Manitoba Institute of Cell Biology, 100 Olivia St., Winnipeg, MB R3E 0V9, Canada.

See "Notes" section following "References."

r the treatment of allergic symptoms
ch as seasonal hayfever or sold over-
e-counter for relief of insomnia or cold
mptoms, bind to H_{1C} over a wide range
potencies (*10*). Therefore, we assessed
e following five H_1-antihistamines for
vivo tumor growth stimulation: 1) lora-
dine (Claritin, Schering-Plough Corp.,
enilworth, N.J.), a nonsedating tricyclic
gent structurally similar to amitriptyline;
) astemizole (Hismanal, Janssen Phar-
aceutica, Piscataway, N.J.), nonsedat-
ng; 3) cetirizine (Reactine, Pfizer
Consumer Health Care, Division of Pfizer
nc., Parsippany, N.J.), sedating; 4) hy-
droxyzine (Atarax, Roerig, Division of
Pfizer Pharmaceuticals, New York,
N.Y.), sedating; and 5) doxylamine (Uni-
som, Pfizer Consumer Health Care; Vicks
NyQuil, Richardson-Vicks Inc., Cincin-
nati, Ohio), sedating.

Reliable in vitro correlative assays could be useful in the preclinical screening of drugs for tumor growth promotion. On the basis of earlier indications, a major aim of this investigation was to evaluate the predictiveness of five in vitro tests. Previously, we reported a correlation among potencies of DPPE and certain H_1- and H_2-antihistamines to compete at H_{1C} sites in rat liver microsomes and to inhibit DNA synthesis in concanavalin A-stimulated mouse spleen cells (*10*) and between potencies of fluoxetine and amitriptyline to stimulate DNA synthesis in C-3 fibrosarcoma cells with a bell-shaped dose–response curve, to inhibit mitogenesis, and to bind to nuclear H_{1C} (*1*). In addition, the demonstration in adrenal microsomes that some H_{1C} sites represent P450 (*8*) suggested that the activity of this family of monooxygenases may be implicated not only in carcinogenesis (*11*), but also in tumor growth promotion.

Therefore, we assessed the potency of each antihistamine in the following in vitro assays: 1) inhibition of [^{3}H]histamine binding to microsomal H_{1C}, 2) inhibition of histamine binding to microsomal P450, 3) inhibition of the P450-catalyzed demethylation of aminopyrine, 4) inhibition of lymphocyte mitogenesis, and 5) stimulation of tumor colony formation. For each agent, the overall cumulative score in the battery of tests was correlated with propensity, at estimated human-equivalent doses, to stimulate the in vivo growth of two transplantable murine tumors (B16F10 melanoma and C-3 fibrosarcoma).

Materials and Methods

In Vitro Assays

Inhibition of [^{3}H]histamine binding to H_{1C} in rat liver microsomes. Microsomal fractions were freshly prepared from livers of adult Sprague–Dawley rats, as described previously (6). [^{3}H]Histamine-binding assays (0.5 mg microsomal protein per milliliter) were performed in a buffer of 10 m*M* HCO_3 containing 0.1 μ*M* $CuCl_2$. [^{3}H]Histamine (5 nmol; Du Pont NEN Research Products, Boston, Mass.) was incubated at room temperature in the dark for 60 minutes with increasing concentrations of cold histamine or of the test agents. The reaction was terminated by centrifugation at 12 000*g* for 15 minutes at 4 °C. Radioactivity was quantitated for replicate samples, and binding data were analyzed using the LIGAND program (*12*) (three to four tests). Under the binding conditions employed, microsomes were found to contain two sites for histamine (K_{d1} [dissociation constant for high-affinity site] = 0.26 ± 0.19 μ*M* [mean ± SEM]; K_{d2} [dissociation constant for low-affinity site] = 36 ± 15 μ*M*).

Inhibition of histamine binding to microsomal P450. The K_i (inhibitory constant) value of each antihistamine for histamine binding to P450 in rat liver microsomes was determined by analyses of difference spectra. A Milton Roy Spectronic 3000 Array Spectrophotometer (Milton Roy Company, New Rochelle, N.Y.) with a computer software-controlled program (Rapidscan) collected and plotted the spectral data. Microsomes were kept frozen (−70 °C) until used, thawed, diluted in potassium phosphate buffer at 4 °C and pH 8.5 (pH optimum for histamine difference spectra), gently homogenized, and preincubated for 30 minutes at 22 °C. Final concentrations of histamine (0.125-1.0 m*M*) and inhibitory doses of antihistamine (20-5000 μ*M*) or buffer (100 μL each) were added to rat liver microsomes (1 mg/mL) in 12 × 75-mm polypropylene tubes (final vol = 1 mL) and incubated for 25 minutes at 22 °C prior to readings. Tissue with or without competitors served as references. The ΔA (amount bound), characterized by the difference between a broad trough at 390–410 nm and a peak at 425–435 nm (*13*), was plotted against the histamine concentrations added in the presence or absence of competitors. K_m (Michaelis–Menten constant), and K_i values were determined from Lineweaver–Burke plots analyzed with the IBM-PC program ENZYME (*14*). Typical mean K_m values ± SEM for histamine binding were 364 ± 22 μ*M* (three to four tests).

Aminopyrine demethylase assay. Rat liver microsomes were prepared by the method of Boobis et al. (*15*). Eppendorf centrifuge tubes (1.5 mL, capped; Brinkmann Instruments, Inc., Westbury, N.Y.) containing a final concentration of 10 μL of aminopyrine (0.1-2.5 m*M*), 50 μL of regenerating system (glucose-6-phosphate, 5 m*M*; glucose-6-phosphate dehydrogenase, 1 U/mL; and nicotinamide-adenine dinucleotide phosphate, 0.3 m*M*), and 940 μL of microsomes (0.5 mg/protein) were incubated for 20 minutes at 37 °C in 50 m*M* Tris buffer (pH 7.4), containing 5 m*M* $MgCl_2$, 1 m*M* EDTA, and 8.0 m*M* nicotinamide. The microsomal suspension was preincubated in 10-mL, 16 × 120-mm conical glass tubes for 15-20 minutes at 20 °C, with or without test agent, and then mixed with substrate and regenerating system. The antihistamines were dissolved in H_2O or ethanol; the ethanol had no effect on enzyme activity. The reaction was stopped with 0.3 mL of 20% trichloroacetic acid. The tubes were centrifuged at 10 000*g* for 10 minutes at 4 °C, and 0.5 mL of supernatant was added to 0.5 mL of NASH reagent (*16*). The mixture was incubated for 10 minutes at 70 °C, then cooled to room temperature. The product (formaldehyde) was determined by absorbance, measured at 412 nm, in a Milton Roy Spectronic 3000 Array Spectrophotometer. Formaldehyde standards (0-0.2 m*M*) in 1.0 mL of buffer and 0.3 mL of 20% trichloroacetic acid were reacted, and the absorbance was determined. Tubes containing no substrate, with or without test agent, were used as references. Typical Michaelis–Menten values were K_m = 0.4 m*M* and V_{max} (maximal velocity) = 4.8 nmol/mg protein per minute. Lineweaver–Burke plots, K_m, and K_i were determined with the IBM-PC program ENZYME by a weighted nonlinear least-squares curve procedure (*17*).

Mitogenesis studies. Fresh spleen cells (5 × 10^5) obtained from 5-week-old BALB/c mice (Charles River, St. Constant, Quebec, Canada) were suspended in RPMI-1640 medium containing 2% fetal calf serum (FCS) (GIBCO BRL, Grand Island, N.Y.), seeded into replicate microwell plates (Nunc, Roskilde, Denmark) to which was added concanavalin A (2.5 μg/mL; Sigma Chemical Co., St. Louis, Mo.), and incubated (37 °C, 95% air, 5% CO_2) in the absence or presence of increasing concentrations of the test agents. Forty-three hours after the addition of concanavalin A, 0.25 nmol [^{3}H]thymidine (6.7 Ci/nmol; ICN Radiopharmaceuticals, Montreal, Quebec) was added to each well. After an additional 5-hour incubation, the cells were washed from the wells onto filter papers with the use of an automated cell sorter. The filters were placed in vials containing 5 mL of scintillation fluid (Readysafe; Beckman Instruments, Inc., Fullerton, Calif.), and radioactivity incorporated into DNA at 48 hours was determined (three assays for each ligand).

Tumor colony growth assay. To assess the effect of the various antihistamines on the growth of B16F10 melanoma cells in vitro, we used suboptimal culture conditions, employing stale (conditioned) medium as described by Vichi and Tritton (*18*) for doxorubicin. B16F10 melanoma cells (1 × 10^6) in 1 mL of conditioned α-minimal essential medium (α-MEM; GIBCO BRL) containing 10% FCS were added to replicate 10-cm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) containing 9 mL of α-MEM (10% FCS) and 0.1 mL of drug or vehicle solution (final drug concentration: 10^{-12}-10^{-4} *M*). After incubation for 24 hours (37 °C, 95% air, 5% CO_2), the cells from each dish were recovered by combining the supernatants from the original solution, the washed solution, and the trypsinizing solution, followed by centrifugation for 10 minutes (700*g* at room temperature). Each cell pellet was resuspended in 10 mL of fresh α-MEM (10% FCS), diluted 1000-fold (final concentration, approximately 10^2 cells/mL), and 1 mL of the cell suspension

added to dishes containing 9 mL of fresh α-
A (10% FCS). Replicate dishes were incubated
10-14 days (37 °C, 95% air, 5% CO_2) until
roscopic (approximately 1 mm in diameter)
nies appeared on the bottom of the dish. The su-
natants were removed, each dish was air dried,
the colonies were stained for 20 minutes with
hylene blue dye and then counted. Percent tumor
ony growth stimulation was calculated by the fol-
ing formula: [(average No. of colonies in repli-
drug-treated cultures)/(average No. of colonies
replicate vehicle-treated cultures)] × 100. The
cent tumor colony stimulation was calculated as
mean value for duplicate cultures at four drug
ncentrations (10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} *M*). Repli-
es were within 5% of the mean value.

tudies of In Vivo Tumor Growth romotion

Tumor studies in mice were carried out in ac-
ordance with institutional guidelines. To test the
producibility of tumor growth stimulation by the
arious drugs, experiments were carried out in two
aboratories, separated by a distance of 750 km; all
rug and vehicle control solutions were made up in
ne laboratory (the laboratory of L. J. Brandes) and
ent under code to the other laboratory (that of R. C.
Warrington). The tumor models employed were
B16F10 melanoma and C-3 fibrosarcoma and were
described in detail previously (*19*); in rodents ad-
ministered daily doses of amitriptyline or fluoxetine,
an excellent correlation has been observed among
tumor stimulation of B16F10 melanoma and C-3
fibrosarcoma in mice and of mammary carcinomas
induced by 7,12-dimethylbenz[*a*]anthracene in rats
(*1*). As a test for tumor growth enhancement by the
H_1-antihistamines, 60-70 female (20 g) mice (strain
C57BL for B16F10 melanoma; strain C3H for C-3
fibrosarcoma) were shaved over the gluteal area.
C57BL mice were given a subcutaneous injection of
5×10^5 tumor cells on day 1. C3H mice were given
a subcutaneous injection of 1×10^6 tumor cells on
day 1. The mice were randomly allocated to treat-
ment groups (10-12 mice for each dose of test
agent), housed in cages of four or five mice each,
fed standard laboratory chow and water ad libitum,
and exposed to cycles of 12 hours of light and 12
hours of dark. Starting 24 hours after the tumor cell
injections, groups of mice received a single, daily
intraperitoneal injection of the test agent at es-
timated human-equivalent dose(s) or vehicle control
for periods ranging from 18 to 21 days, and then the
mice were killed. The daily recommended human
dose of loratadine, astemizole, or cetirizine is 10 mg
(approximately 6 mg/m^2) (*20,21*); for hydroxyzine,
the average daily human dose ranges from 25 to 200
mg (15-120 mg/m^2) (*20*). The daily recommended
dose of doxylamine for humans ranges from 7.5 to
30 mg (4-16 mg/m^2) (*21*). Therefore, to test for
tumor growth stimulation at estimated human-
equivalent doses, we gave groups of mice the fol-
lowing dose, or range of doses, of the test agents:
loratadine, 6 mg/m^2; astemizole, 1.5, 3, 4, 6, and 24
mg/m^2; cetirizine, 6, 12, and 24 mg/m^2; hydroxy-
zine, 40 and 60 mg/m^2; and doxylamine, 4, 8, and 16
mg/m^2. Control animals received a daily intra-
peritoneal injection of a solution containing vehicle
alone. (Saline was used to dissolve hydroxyzine,
cetirizine, and doxylamine; 0.4 *M* tartaric acid was
used to dissolve astemizole and loratadine.) On days
18-21 of treatment, the mice were killed by CO_2 as-
phyxiation, the tumors dissected and weighed, and
tumor wet weights compared among groups. All
cages were coded, and measurement of tumors was
done by a blinded procedure.

Statistical Analysis

Statistical differences among tumor wet weights
in treatment groups were assessed using Student's
two-tailed *t* test or, where indicated in the text,
Fisher's exact test. Correlations between the rank
order of the antihistamines in the five in vitro assays
and the rank order to promote tumors in vivo were
analyzed for significance using Spearman's coeffi-
cient of rank correlation (*22*).

Results

Based on rank order of potency in each
of the five assays (maximum cumulative
score = 25 points), the overall ranking
(Table 1) was as follows: loratadine = as-
temizole > hydroxyzine > doxylamine >
cetirizine.

The results of administration (18-21
days) of the antihistamines on tumor
growth in vivo are shown in Fig. 1.
Loratadine (Fig. 1, A) at a dose of 6
mg/m^2, equivalent to the prescribed dose
of 10 mg/d in humans (*20*), stimulated the
growth of B16F10 melanoma (150% ±
5% of control [mean ± SEM]; $P<.001$)
and of C-3 fibrosarcoma (390% ± 20% of
control; $P<.001$). Astemizole (Fig. 1, B),
tested over a dose range of 1.5-24 mg/m^2
per day, stimulated the growth of both
melanoma and fibrosarcoma with a bell-
shaped dose–response curve; however,
enhancement of C-3 fibrosarcoma growth
(184% ± 25% of control; $P<.001$) oc-
curred at 6 mg/m^2, equivalent to the usual
human dose of 10 mg/d (*21*), whereas en-
hancement of melanoma growth (171% ±
9% of control; $P<.001$) occurred at 3
mg/m^2, and 6 mg/m^2 was mildly in-
hibitory. Hydroxyzine (Fig. 1, C) at a
dose of 60 mg/m^2 also stimulated the
growth of B16 melanoma (194% ± 15%
of control; $P<.001$); stimulation of C-3
fibrosarcoma (125% ± 13% of control)
was not significant, but the number of
large tumors (14 of 22; mean wet weights
= 164% ± 16% of control) in animals
treated with hydroxyzine at a dose of 40-
60 mg/m^2 was greater than that in
animals receiving vehicle alone (seven of
19; mean wet weights = 155% ± 13% of
control; $P = .08$; Fisher's exact test). In
contrast, cetirizine (Fig. 1, D), which dif-
fers from hydroxyzine by the single side-
chain substitution of a terminal acid
group for an alcohol, had no statistically
significant effect on the growth of either
tumor when it was administered over a
human-equivalent dose range of 6-24
mg/m^2. Doxylamine at a dose of 4-16
mg/m^2 did not increase tumor growth; at
its highest concentration, doxylamine in-
hibited the growth of both tumors by
30%-40% ($P<.001$; data not shown).

Drug-induced stimulation of melanoma
colony growth in vitro (Fig. 2) correlated
with stimulation of melanoma growth in
vivo. Except for loratadine, a bell-shaped
dose–response curve,[1] maximal between
10^{-8} *M* and 10^{-10} *M*, was observed (Fig.
2). The three drugs that stimulated mela-
noma growth in vivo—astemizole, hydrox-
yzine, and loratadine—were stimulatory
in vitro, although the relative potencies of
astemizole and hydroxyzine were re-
versed; doxylamine and cetirizine were
much weaker.

Overall, a significant correlation (r =
.97; $P<.02$) was observed between the
rank order of potency of the antihis-
tamines in all five in vitro assays and
their rank order to enhance tumor growth
in vivo (Table 2).

Discussion

The data demonstrate that, individually
or combined, the in vitro assays predicted
the propensity of each H_1-antihistamine
to stimulate cancer growth in vivo. The
almost perfect correlation among the rank
order to inhibit [^{3}H]histamine binding to
H_{1C} in microsomes, histamine binding to
microsomal P450, and P450-catalyzed
demethylation of aminopyrine (Table 1)
supports our previous demonstration (*8*)
in adrenal tissue that at least some
proportion of H_{1C} in microsomes repre-
sents mono-oxygenases. Histamine and
some other imidazoles are endogenous
heme-binding substrates for P450 (*23*);
thus, the observation that the rank order
of drug potency to inhibit histamine bind-
ing and P450 enzyme activity corre-
sponded to the rank order of potency to
inhibit lymphocyte mitogenesis and
propensity to stimulate tumor growth sug-
gests that intracellular histamine may
modulate the activity of certain P450 en-
zymes which, in turn, modulate cell
proliferation (*24*). For example, the in-

Table 1. Potency of five H_1-antihistamines in five in vitro assays*

	[^{3}H]Histamine binding to H_{1C}			Histamine binding to P450		Aminopyrine demethylase		Mitogenesis		Tumor colony growth		
H_1-antihistamine	K_{i1}, μM*	K_{i2}, μM*	Rank†	K_i, μM*	Rank†	IC_{50}, μM*	Rank†	IC_{50}, μM*	Rank†	% stimulation	Rank†	Score†
Loratadine	2 ± 1	381 ± 114	2	13.5 ± 1.5	1	3.1 ± 0.3	1	1.0 ± 0.5	1	21	3	22
Astemizole	2.8 ± 2.2	36 ± 5.5	1	31 ± 1.7	2	27 ± 2	2	2.0 ± 0.2	2	35	1	22
Hydroxyzine	1.2 ± 0.4	2080 ± 825	3	62 ± 5.6	3	38 ± 8	3	12 ± 1	3	30	2	16
Doxylamine	0.8 ± 0.3	>3500	4	142 ± 35	4	73 ± 8	4	70 ± 5	4	9	4	10
Cetirizine	NB	NB	5	1537 ± 186	5	762 ± 85	5	160 ± 10	5	9	4	6

*Values = means ± SEM (three to six assays for each of the five tests). K_{i1} = inhibitory constant for high-affinity site; K_{i2} = inhibitory constant for low-affinity site; IC_{50} = concentration that causes 50% inhibition; NB = no binding detected.

†Scoring in each assay by rank as follows: rank 1 = 5 points; 2 = 4 points; 3 = 3 points; 4 = 2 points; 5 = 1 point.

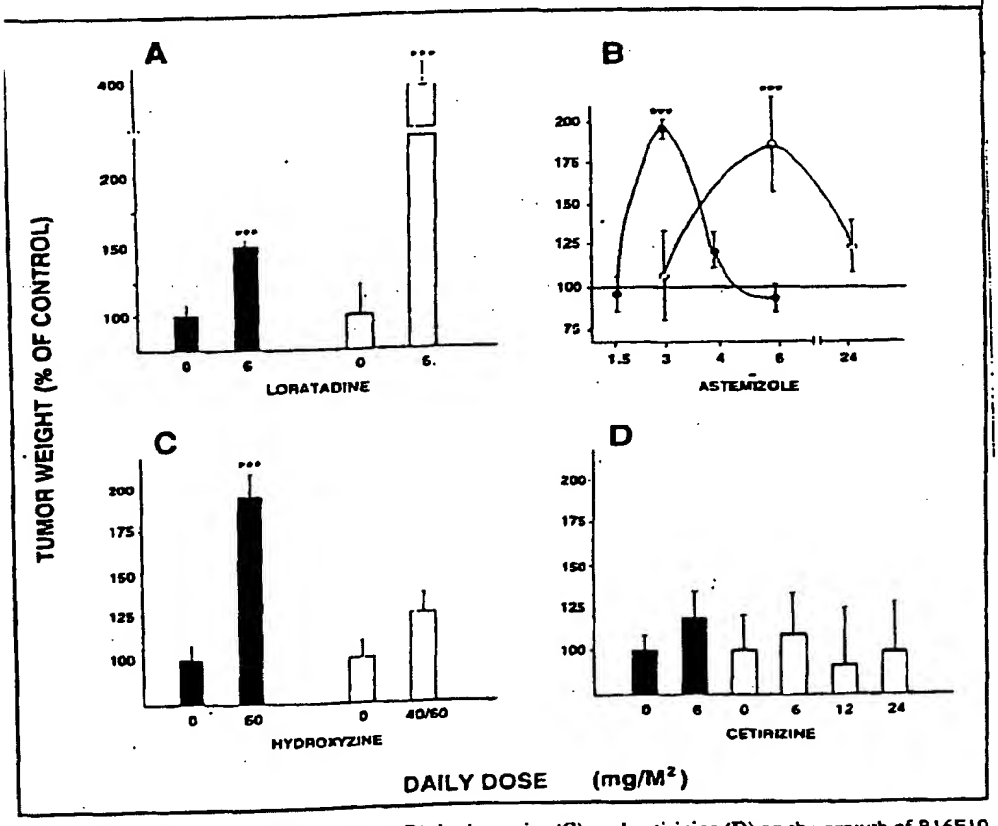


Fig. 1. Effect of loratadine (A), astemizole (B), hydroxyzine (C), and cetirizine (D) on the growth of B16F10 melanoma (■ = A, C, and D; ● = B) and C-3 fibrosarcoma (□ = A, C, and D; ○ = B). ***P<.001; for loratadine, number of mice = 10-11; for hydroxyzine, number of mice = 19-22; for cetirizine, number of mice = 10-21 for each concentration; for astemizole, number of mice = 10 for each concentration (B16F10 melanoma) and number of mice = 20 for each concentration (C-3 fibrosarcoma). Bars = SEM.

duction of various cytochromes P450 and of histidine decarboxylase, the histamine-forming enzyme, is associated with both tumor growth and the mitogenic response (*10*,*25*). A comprehensive review (*26*) of many studies concluded that drug-induced modification of tumorigenesis is associated with the altered expression of a number of enzymes, including cytochromes P450.

That the concentrations of the antihistamines to modulate growth were lower than those required to inhibit histamine binding to P450 may signify a more potent interaction with specific cell P450s that control proliferation and/or are induced in proliferating lymphocytes (*25*) and in malignant cells (*27*), whereas the binding potency in liver microsomes might reflect overall affinity for a composite of P450 enzymes. Similarly, the rank order of potency to inhibit [^{3}H]histamine binding to microsomal H_{1C} at low, but not high, affinity sites (Table 1) correlated with the rank order of potency in the proliferative assays, suggesting that the lower affinity sites represent, at least in part, binding to P450. The observation of a reasonable correspondence between the absolute concentrations of drugs to inhibit aminopyrine demethylase and those to inhibit mitogenesis suggests that the P450 isoenzymes that metabolize aminopyrine are relatively closely linked to mitogenic processes.

As a class, drugs that promote tumor growth tend to be immunosuppressive (*26*,*28*); likewise, the potency of the five antihistamines to inhibit lymphocyte mitogenesis correlated highly with their propensity to stimulate tumor growth in vivo. Thus, this simple and highly reproducible test, requiring little in the way of sophisticated laboratory equipment, may be the easiest assay with which to screen potential tumor growth promoters. For example, DPPE, fluoxetine, and amitriptyline are potent inhibitors of mitogenesis (IC_{50} = 0.1 μM, 0.75 μM, and 2.5 μM, respectively) and accelerate tumor growth in vivo (*1*,*5*). Similarly, the same correlation shown now for astemizole, loratadine, and hydroxyzine raises concerns about other antihistamines that potently (IC_{50}, ≤10 μM) inhibit mitogenesis, including prochlorperazine (Compazine, SmithKline Beecham, Philadelphia, Pa.; IC_{50} = 1 μM), promethazine (Phenergan, Wyeth-Ayerst Laboratories, Philadelphia, Pa.; IC_{50} = 5 μM), and terfenadine[2] (Seldane, Marion Merrell Dow Inc.; IC_{50} = 10 μM).

We recognize that the predictor tests have been applied to a small number of

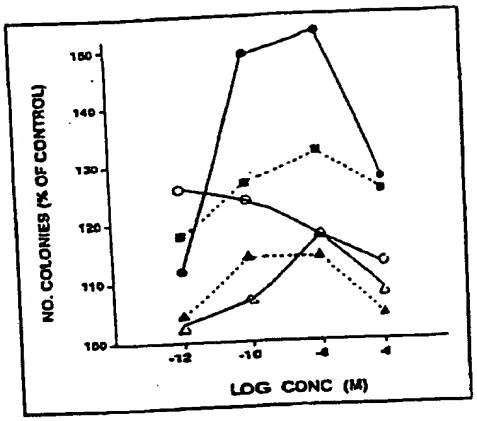


Fig. 2. Stimulation of B16F10 melanoma colony growth in vitro by loratadine (○), astemizole (●), hydroxyzine (■), doxylamine (▲), and cetirizine (△), as described in "Materials and Methods" section.

Table 2. Correlation between rank order of potency of antihistamines in five in vitro assays and rank order to promote tumors in vivo*

In vitro assay	r†
[^{3}H]Histamine binding	.95‡
Histamine binding to P450	.95‡
Aminopyrine demethylase	.95‡
Mitogenesis	.81
Tumor colony growth	.97‡
Cumulative score—five assays	

*Potency to promote tumors in vivo was ranked according to whether the drug significantly stimulated the growth of C-3 fibrosarcoma and B16F10 melanoma. Based on this criterion, loratadine and astemizole were each ranked first (significant promotion of both tumors); hydroxyzine ranked third behind loratadine and astemizole (significant promotion of B16 melanoma), and doxylamine and cetirizine each ranked fourth (no significant promotion of either tumor). Thus, rank order in each in vitro assay and rank order of cumulative scores in all five assays (Table 1) were compared with the rank order 1, 1, 3, 4, and 4 in the in vivo tumor promotion assays.

†Spearman's coefficient of rank correlation.

‡$P<.02$.

compounds. Moreover, drug administration was by the intraperitoneal route only and may have yielded results different from those obtained with oral administration, the latter characterized by variable absorption and first-pass liver metabolism.[3] Also, as exemplified by the bell-shaped dose–response curves and the differing effects of astemizole and hydroxyzine on the growth of B16F10 melanoma and C-3 fibrosarcoma, tumor promotion may depend on both the tumor type and the drug dose; a panel of several transplantable tumor lines or additional models, such as 7,12-dimethylbenz[*a*]anthracene-induced carcinogenesis (*1*,*2*,*5*) or human tumors implanted into nude mice, might best expose the propensity of drugs, administered over a wide human-equivalent dose range, to stimulate tumor growth.

Although the potential for carcinogenicity has received considerable attention in preclinical drug testing in rodents, the propensity of pharmaceuticals to enhance the growth of existing tumors or the development of malignancy induced by chemical or viral initiators has been neglected. While caution must be exercised in extrapolating our data from rodents to humans, we believe that epidemiologic studies (*29*) may further contribute to an understanding of the potential risks that our laboratory findings have exposed.

References

(*1*) Brandes LJ, Arron RJ, Bogdanovic RP, et al: Stimulation of malignant growth in rodents by antidepressant drugs at clinically relevant doses. Cancer Res 52:3796-3800, 1992

(*2*) Hilakivi-Clarke L, Wright A, Lippman ME: Neonatal antidepressant treatment promotes DMBA-induced mammary tumor growth. Proc Am Assoc Cancer Res 34:184, 1993

(*3*) Ilishi H, Tatsuta M, Baba M, et al: Enhancement by the tricyclic antidepressant, desipramine, of experimental carcinogenesis in rat colon induced by azoxymethane. Carcinogenesis 14:1837-1840, 1993

(*4*) Brandes LJ, LaBella FS: Histamine and calcium are independently regulated intracellular mediators of lymphocyte mitogenesis. Biochem Biophys Res Commun 182:786-793, 1992

(*5*) Brandes LJ, Beecroft WA, Hogg GR: Stimulation of in vivo tumor growth and phorbol ester-induced inflammation by *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine HCl, a potent ligand for intracellular histamine receptors. Biochem Biophys Res Commun 179:1297-1304, 1991

(*6*) Brandes LJ, Bogdanovic RP, Cawker MD, et al: Histamine and growth: interaction of antiestrogen binding site ligands with a novel histamine site that may be associated with calcium channels. Cancer Res 47:4025-4031, 1987

(*7*) Brandes LJ, LaBella FS: Identification of intracellular histamine receptors (H_{IC}) that regulate cell proliferation. *In* Histamine in Normal and Cancer Cell Proliferation (Garcia-Caballero M, ed). Oxford: Pergamon Press, 1993, pp 31-41

(*8*) LaBella FS, Queen G, Glavin G, et al: H_3 receptor antagonist, thioperamide, inhibits adrenal steroidogenesis and histamine binding to adrenocortical microsomes and binds to cytochrome P450. Br J Pharmacol 107:161-164, 1992

(*9*) Fendl KC, Zimniski SJ: Role of tamoxifen in the induction of hormone-independent rat mammary tumors. Cancer Res 52:235-237, 1992

(*10*) Brandes LJ, Davie JR, Paraskevas F, et al: The antiproliferative potency of histamine antagonists correlates with inhibition of binding of ^{3}H-histamine to novel intracellular receptors (H_{IC}) in microsomal and nuclear fractions of rat liver. Agents Actions Suppl 33:325-342, 1991

(*11*) Conney AH: Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G H A Clowes Memorial Lecture. Cancer Res 42:4875-4917, 1982

(*12*) Munson PJ, Rodbard D: LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220-239, 1980

(*13*) Jeffcoate CR: Measurement of substrate and inhibitor binding to microsomal cytochrome P450 by optical-difference spectroscopy. Methods Enzymol (C) 52:258-279, 1978

(*14*) Lutz RA, Bull C: Rodbard computer analysis of enzyme-substrate inhibitor kinetic data with automatic model selection using IBM-PC compatible microcomputers. Enzyme 36:197-206, 1986

(*15*) Boobis AR, Brodie MJ, Kahn GC, et al: Monooxygenase activity of human liver in microsomal fractions of needle biopsy specimens. Br J Clin Pharmacol 9:11-19, 1980

(*16*) Nash T: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J 55:416-421, 1953

(*17*) Lutz RA, Rodbard D: Computer Analysis of Enzyme-Substrate-Inhibitor Kinetic Data. Bethesda, Md: NIH, 1986

(*18*) Vichi P, Tritton TR: Stimulation of growth in human and murine cells by Adriamycin. Cancer Res 49:2679-2682, 1989

(*19*) Brandes LJ, LaBella FS, Warrington RC: Increased therapeutic index of antineoplastic drugs in combination with intracellular histamine antagonists. J Natl Cancer Inst 83:1329-1336, 1991

(*20*) Krogh CME, ed: Compendium of Pharmaceuticals and Specialties. Ottawa: Canadian Pharmaceuticals Assoc, 1993, pp 245-246, 1030

(*21*) McEnvoy GK, ed: American Hospital Formulary Drug Information. Bethesda, Md: American Society of Hospital Pharmacists, Inc, 1993, pp 1-32

(*22*) Steel R, Torrie JH: Non-parametric statistics. *In* Principles and Procedures of Statistics. New York: McGraw-Hill, 1960, p 409

(*23*) Morris CQ, Tucker GT, Crewe HK, et al: Histamine inhibition of mixed function oxidase

for this study. Personal interviews solicited information on age, income, marital status, place of birth, education, health insurance coverage, Pap smear- and mammogram-screening practices, and six questions relating to social network. A social network score was assigned to each woman by sum-

Prevention), L. Lloyd, N. Weiss, T. Rainbolt (Cancer Registry Division), Texas Department of Health, Austin.

L. Pulley, Center for Health Promotion Research and Development, University of Texas Houston Health Science Center.

Correspondence to: Lucina Suarez, M.S., Disease Prevention, Texas Department of Health, 1100 West 49th St., Austin, TX 78756.

See "Notes" section following "References."